THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITORIAL BOARD

RUDOLPH J. ANDERSON
REGINALD M. ARCHIBALD
ERIC G. BALL
ARNOLD KENT BALLS
KONRAD BLOCH
GEORGE BOSWORTH BROWN
HERBERT E. CARTER
WALDO E. COHN
CARL F. CORI
EDWARD A. DOISY
JOHN T. EDSALL
JOSEPH S. FRUTON
WENDELL H. GRIFFITH
SAMUEL GURIN
A. BAIRD HASTINGS
LESLIE HELLERMAN
ALBERT L. LEHNINGER
STANFORD MOORE
HANS NEURATH
SEVERO OCHOA
ESMOND E. SNELL
WARREN M. SPERRY
DEWITT STETTEN, JR.
EDWARD L. TATUM
HUBERT B. VICKERY
VINCENT DU VIGNEAUD
O. WINTERSTEINER

VOLUME 215
BALTIMORE
1955

XUM

COPYRIGHT, 1955
BY
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

PUBLISHED AT YALE UNIVERSITY FOR
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, INC.
BALTIMORE 2, U. S. A.

QP
501
J7
Vol. 215

CONTENTS OF VOLUME 215

No. 1, July, 1955

PAGE

Korn, Edward D. Clearing factor, a heparin-activated lipoprotein lipase. I. Isolation and characterization of the enzyme from normal rat heart 1

Korn, Edward D. Clearing factor, a heparin-activated lipoprotein lipase. II. Substrate specificity and activation of coconut oil 15

Tolbert, N. E. Formic acid metabolism in barley leaves 27

Nyc, Joseph F., and Zabin, Irving. The formation of serine from pyruvate .. 35

Sime, John T., and Johnson, B. Connor. Metabolism of methionine-$C^{14}H_3$ in the chick. II. Distribution of C^{14} in the urinary uric acid molecule 41

Dannenberg, Arthur M., Jr., and Smith, Emil L. Proteolytic enzymes of lung 45

Dannenberg, Arthur M., Jr., and Smith, Emil L. Action of Proteinase I of bovine lung. Hydrolysis of the oxidized B chain of insulin; polymer formation from amino acid esters 55

Smith, Emil L., Kimmel, J. R., Brown, Douglas M., and Thompson, E. O. P. Isolation and properties of a crystalline mercury derivative of a lysozyme from papaya latex 67

Webster, George C., and Varner, J. E. Aspartate metabolism and asparagine synthesis in plant systems 91

Rose, William C., Coon, Minor J., Lockhart, Haines B., and Lambert, G. Frederick. The amino acid requirements of man. XI. The threonine and methionine requirements 101

Borsook, Henry, Abrams, Adolph, and Lowy, Peter H. Fructose-amino acids in liver: stimuli of amino acid incorporation *in vitro* 111

Hayaishi, Osamu. Enzymatic decarboxylation of malonic acid 125

Bernstein, I. A., Lentz, Kenneth, Malm, Mignon, Schambye, Per, and Wood, Harland G. Degradation of glucose-C^{14} with *Leuconostoc mesenteroides;* alternate pathways and tracer patterns 137

Ashmore, James, Renold, Albert E., Nesbett, Frances B., and Hastings, A. Baird. Studies on carbohydrate metabolism in rat liver slices. V. Glycerol metabolism in relation to other substrates in normal and diabetic tissue 153

Hess, E. L., Campbell, Mildred, and Herranen, Ailene. A protease present in extracts of a lymphatic organ 163

Villee, Claude A. An estradiol-induced stimulation of citrate utilization by placenta 171

Shuster, Louis, and Kaplan, Nathan O. The preparation and properties of 3′-triphosphopyridine nucleotide 183

Shuster, Louis, Kaplan, Nathan O., and Stolzenbach, Francis E. The reaction of pyridine nucleotides with trifluoroacetic acid anhydride 195

Thannhauser, S. J., Fellig, Josef, and Schmidt, Gerhard. The structure of cerebroside sulfuric ester of beef brain 211

Buston, H. W., and Bishop, Jillian. Synthetic α-amino-β-hydroxycaproic acids 217

Siekevitz, Philip, and Potter, Van R. Biochemical structure of mitochondria. I. Intramitochondrial components and oxidative phosphorylation .. 221

XUM

SIEKEVITZ, PHILIP, and POTTER, VAN R. Biochemical structure of mitochondria. II. Radioactive labeling of intramitochondrial nucleotides during oxidative phosphorylation 237
FUTTERMAN, SIDNEY, and ROE, JOSEPH H. The identification of ribulose and L-xylulose in human and rat urine 257
SERLIN, IRVING, and COTZIAS, GEORGE C. Microdiffusion of acetic acid as an assay for acetylcholinesterase 263
PRIVAT DE GARILHE, M., and LASKOWSKI, M. Study of the enzymatic degradation of deoxyribonucleic acid by two different deoxyribonucleodepolymerases 269
RAACKE, I. D., and LI, CHOH HAO. Corticotropins (ACTH). VI. Isoelectric point of α-corticotropin as determined by zone electrophoresis on starch ... 277
BOTT, P. A. The concentration of potassium in glomerular urine of *Necturi* .. 287
OKUHARA, EIJI, and NAKAYAMA, TEISHI. Studies on the conjugated lipides. VI. On the structure of inositol phospholipide 295
HARRISON, W. H., BOYER, P. D., and FALCONE, A. B. The mechanism of enzymic phosphate transfer reactions 303
STEWART, CHARLES J., CHELDELIN, VERNON H., and KING, TSOO E. Synthesis and biological activities of pantetheine analogues. XVI. Pantothenic acid studies 319
UDENFRIEND, SIDNEY, WEISSBACH, HERBERT, and CLARK, CARROLL T. The estimation of 5-hydroxytryptamine (serotonin) in biological tissues 337
PIHL, ALEXANDER, and FRITZSON, PER. The catabolism of C^{14}-labeled β-alanine in the intact rat 345
PERLMAN, D. Spectrophotometric method for the determination of 5-keto-D-gluconic acid 353
GABRIO, BEVERLY WESCOTT, HENNESSEY, MARION, THOMASSON, JOAN, and FINCH, CLEMENT A. Erythrocyte preservation. IV. *In vitro* reversibility of the storage lesion 357
HENDERSON, L. M., KOSKI, RUTH E., and D'ANGELI, F. The rôle of riboflavin and vitamin B_6 in tryptophan metabolism 369
MALEY, GLADYS FELDOTT, and LARDY, HENRY A. Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rat livers 377
KORNBERG, ARTHUR, LIEBERMAN, IRVING, and SIMMS, ERNEST S. Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate 389
LIEBERMAN, IRVING, KORNBERG, ARTHUR, and SIMMS, ERNEST S. Enzymatic synthesis of pyrimidine nucleotides. Orotidine-5'-phosphate and uridine-5'-phosphate 403
KORNBERG, ARTHUR, LIEBERMAN, IRVING, and SIMMS, ERNEST S. Enzymatic synthesis of purine nucleotides 417
LIEBERMAN, IRVING, KORNBERG, ARTHUR, and SIMMS, ERNEST S. Enzymatic synthesis of nucleoside diphosphates and triphosphates 429
MEISTER, ALTON, LEVINTOW, LEON, GREENFIELD, ROBERT E., and ABENDSCHEIN, PATRICIA A. Hydrolysis and transfer reactions catalyzed by ω-amidase preparations 441

No. 2, AUGUST, 1955

BLOOM, BEN, EISENBERG, FRANK, JR., and STETTEN, DEWITT, JR. Glucose catabolism in liver slices via the phosphogluconate oxidation pathway 461

BLOOM, BEN. Fraction of glucose catabolized via the Embden-Meyerhof pathway: alloxan-diabetic and fasted rats 467
TONG, W., and CHAIKOFF, I. L. Metabolism of I^{131} by the marine alga, *Nereocystis luetkeana* 473
WOOLLEY, D. W., SCHAFFNER, G., and BRAUN, ARMIN C. Studies on the structure of the phytopathogenic toxin of *Pseudomonas tabaci* 485
BUEDING, ERNEST, and MACKINNON, JOAN A. Hexokinases of *Schistosoma mansoni* 495
BUEDING, ERNEST, and MACKINNON, JOAN A. Studies of the phosphoglucose isomerase of *Schistosoma mansoni* 507
GUARINO, ARMAND J., and SABLE, HENRY Z. Studies on phosphomutases. II. Phosphoribomutase and phosphoglucomutase 515
FISHMAN, WILLIAM H., and GREEN, SIDNEY. Microanalysis of glucuronide glucuronic acid as applied to β-glucuronidase and glucuronic acid studies 527
MORGAN, JOSEPH F., and MORTON, HELEN J. Studies on the sulfur metabolism of tissues cultivated *in vitro*. I. A critical requirement for L-cystine 539
MATSUO, YOSHIHIKO, and GREENBERG, DAVID M. Metabolic formation of homoserine and α-aminobutyric acid from methionine 547
NIELSEN, SIGURD OLAF, and LEHNINGER, ALBERT L. Phosphorylation coupled to the oxidation of ferrocytochrome *c* 555
BORGSTRÖM, BENGT, SUDDUTH, HERSCHEL C., and LEHNINGER, ALBERT L. Phosphorylation coupled to reduction of cytochrome *c* by β-hydroxybutyrate 571
SINSHEIMER, ROBERT L. The action of pancreatic deoxyribonuclease. II. Isomeric dinucleotides 579
EISENSTEIN, ALBERT B. Metabolism of cortisone by rat liver homogenate 585
RACHELE, JULIAN R., KUCHINSKAS, EDWARD J., KRATZER, F. HOWARD, and DU VIGNEAUD, VINCENT. Hydrogen isotope effect in the oxidation *in vivo* of methionine labeled in the methyl group 593
WRISTON, JOHN C., JR., LACK, LEON, and SHEMIN, DAVID. The mechanism of porphyrin formation. Further evidence on the relationship of the citric acid cycle and porphyrin formation 603
SHEMIN, DAVID, RUSSELL, CHARLOTTE S., and ABRAMSKY, TESSA. The succinate-glycine cycle. I. The mechanism of pyrrole synthesis 613
HUFFMAN, MAX N., and LOTT, MARY HARRIET. 16-Substituted steroids. XII. Estradiol-3,16α 627
HUFFMAN, MAX N., and LOTT, MARY HARRIET. 16-Substituted steroids. XIII. Androstan-3β,16β-diol and androstan-3β,16α-diol 633
WOLFE, R. S., and O'KANE, D. J. Cofactors of the carbon dioxide exchange reaction of *Clostridium butyricum* 637
LENTZ, KENNETH, and WOOD, HARLAND G. Synthesis of acetate from formate and carbon dioxide by *Clostridium thermoaceticum* 645
TROLL, WALTER, and LINDSLEY, JOHN. A photometric method for the determination of proline 655
BEATTY, CLARISSA H., and WEST, EDWARD S. Effect of succinic and malic acids and fructose on ketosis in alloxan-diabetic rats 661
JORDAN, WILLIAM J., and GRAY, IRVING. The biochemical response to trauma. I. Nucleotide changes in tourniquet shock 669
TOUSTER, OSCAR, HUTCHESON, RUTH M., and RICE, LOUISE. The influence of D-glucuronolactone on the excretion of L-xylulose by humans and guinea pigs 677

DEISS, WILLIAM P., and LEON, ARTHUR S. Mucopolysaccharides of heart valve mucoprotein 685
MOUNTER, L. A., DIEN, LIEN TIEN H., and CHANUTIN, ALFRED. The distribution of dialkylfluorophosphatases in the tissues of various species 691
MOUNTER, L. A., BAXTER, ROBERT F., and CHANUTIN, ALFRED. Dialkylfluorophosphatases of microorganisms 699
MOUNTER, L. A. The complex nature of dialkylfluorophosphatases of hog and rat liver and kidney 705
FORCHIELLI, ENRICO, ROSENKRANTZ, HARRIS, and DORFMAN, RALPH I. Metabolism of 11-deoxycortisol *in vitro* 713
LARNER, JOSEPH, and MCNICKLE, C. M. Gastrointestinal digestion of starch. I. The action of oligo-1,6-glucosidase on branched saccharides 723
AISENBERG, A. C., and POTTER, VAN R. Effect of fluoride and dinitrophenol on acetate activation in kidney and liver homogenates 737
FRESCO, JACQUES R., and WARNER, ROBERT C. Application of the isotope derivative method to the analysis of pyrimidines 751
SPITNIK, PNINA, LIPSHITZ, RAKOMA, and CHARGAFF, ERWIN. Studies on nucleoproteins. II. Deoxyribonucleic acid complexes with basic polyelectrolytes and their fractional extraction 765
VISWANATHA, T., and LIENER, IRVIN E. The inhibition of trypsin. III. Influence of urea 777
CRAMPTON, CHARLES F., MOORE, STANFORD, and STEIN, WILLIAM H. Chromatographic fractionation of calf thymus histone 787
NAKADA, HENRY I., MORITA, TOSHIKO N., and WICK, ARNE N. Studies on the relationships between insulin, glucosamine, and glucose in rat diaphragms .. 803
PRUSOFF, WILLIAM H. Studies on the mechanism of action of 6-azathymine. I. Biosynthesis of the deoxyriboside 809
LORBER, VICTOR, and COOK, MARGARET. The metabolism of *n*-butyrate-C^{14} by mammalian heart muscle 823
SMITH, LUCILE. Reactions of cytochromes *a* and a_3. I. Studies of oxidation and reduction of the pigments in a purified preparation 833
SMITH, LUCILE. Reactions of cytochromes *a* and a_3. II. Studies with *Micrococcus pyogenes* var. *albus* and *Bacillus subtilis* 847
INDEX TO VOLUME 215 859

Florisil

is a highly selective adsorbent . . .

A hard, porous, white granular synthetic adsorbent, produced in a variety of mesh classifications, Florisil is being successfully used in the most difficult separations of organic compounds.

Originally recognized for chromatographic adsorption, Florisil is finding a variety of uses in research and manufacture.

This high purity adsorbent is available in quantity as desired, in containers from one to fifty pounds.

Your inquiry will be accorded strict confidence and prompt response.

• • • • •

We also have available Fuller's earth and bauxite-based adsorbents for all types of adsorption filtration including economical decolorization, dehydration and purification.

FLORIDIN COMPANY

ADSORBENTS
DESICCANTS
DILUENTS

DEPT. W, BOX 998, TALLAHASSEE, FLA.

* 2,4-Diamino-6,7-dimethyl Pteridine

* Acetyl Glucosamine

* 8-Azaxanthine

* Dicetyl Phosphate

* 2-Thioxanthine

* Gramine

SEND FOR CATALOG LISTING MANY FINE BIOCHEMICALS AND ORGANICS

Krishell

LABORATORIES, INC.

1735 S. E. Powell Blvd.
Portland 2, Oregon

Concise, clear, direct

PRACTICAL METHODS IN *Biochemistry*

SIXTH EDITION

By Frederick C. Koch & Martin E. Hanke

Combines the quantitative methods of the chemist with the comparative modes of study of the biologist.

Designed to develop quantitative sense and a critical attitude toward the interpretation of laboratory methods and observations.

"An invaluable text for biochemical procedures .... Both clinicians and biochemists should include it in their libraries as a standard reference."—*Am. J. Clin. Path.*

550 pp. 28 figs. $5.00

The Williams & Wilkins Co.

Mt. Royal and Guilford Aves. Baltimore 2, Md.

Index to Volumes 151–175

PRICE $2.00

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Indexes to Volumes 26–50, Volumes 51–75, Volumes 76–100, $1.00 each

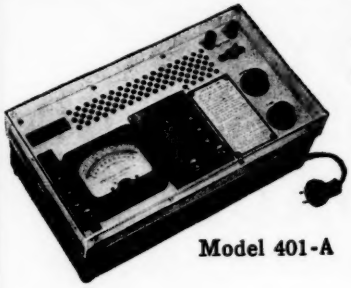
Indexes to Volumes 101–125 and Volumes 126–150 $2.00 each

Please send Index to Volumes_____of The Journal of Biological Chemistry

NAME_____

ADDRESS_____

Orders and remittances (made payable to The American Society of Biological Chemists, Inc.) should be forwarded to The Williams and Wilkins Company, Mt. Royal and Guilford Avenues, Baltimore 2, Maryland.



Model 401-A

LUMETRON Clinical

PHOTOELECTRIC COLORIMETER

for 82 determinations

including

Albumin
Amino Acids
Amylase
Bilirubin
Blood Volume
Bromide
Bromsulphalein
Calcium
Carbon Dioxide
Chloride
Cholesterol
Cholesterol Esters
Creatine
Creatinine
Ethyl Alcohol
Galactose
Gamma Globulin
Globulin
Glucose
Hemoglobin
Hydrogen Ion
Icteric Index
Iron
Lactic Acid
Lead
Lipids
Magnesium
N.P.N.
Phenols
P.S.P.
Phosphatase
Phosphorus
Potassium
Protein
RBC Count
Salicylates
Sodium
Sulfa Comp.
Thiocyanate
Thymol Turb.
Urea Nitrogen
Uric Acid
Urobilinogen
Vitamin A
Vitamin C
Zinc Sulphate
17-Ketosteroids

Write for descriptive Bulletin No. 406. also for free copy of 12-page Introduction to Clinical Photoelectric Colorimetry.

150-page Reference Book available separately, price $3.00.

PHOTOVOLT CORPORATION

95 Madison Avenue • New York 16, N. Y.

THE JOURNAL OF ENDOCRINOLOGY

MAY 1955, VOL. 12, NO. 3

Quantitative studies on the hormonal induction of oestrus in spayed ewes, T. J. ROBINSON

The effects of growth hormone and corticotrophin upon the adrenal weight and adrenocortical mitotic activity in the hypophysectomized rat, D. B. CATER and M. P. STACK-DUNNE

Measurement of the early thyroid clearance of radioiodine as a clinical test, J. COENEGRACHT and R. FRASER

Variations in plasma protein mass during the menstrual cycle of the Chacma baboon (*Papio ursinus*), S. COHEN

Responses of hypophysectomized rats to stress, F. E. BADRICK, R. W. BRIMBLECOMBE and M. REISS

A method for the determination of urinary pregnanediol, A. KLOPPER, EILEEN A. MICHIE and J. B. BROWN

The effect of ACTH and cortisone on the survival of skin homografts and on the adrenal glands in monkeys (*Macaca mulatta*), P. L. KROHN

Proceedings of the Society for Endocrinology.

Subcription Rate: $12.00 per volume of 4 parts

Cambridge University Press

32 East 57th Street NEW YORK 22, N. Y.

XUM

Emphasizes histology's intimate relation to physiology and pathology

BAILEY'S TEXT-BOOK OF HISTOLOGY

THIRTEENTH EDITION

Revised by **PHILIP E. SMITH, Ph.D., Sc.D.**, and **WILFRED M. COPENHAVER, Ph.D.** With the assistance of **Dorothy D. Johnson, Ph.D.**

The ideal text-book because it is complete and comprehensive—since a thorough knowledge of normal histology is essential for the understanding of the altered structure seen in the various conditions of disease.

Includes full discussion of the physiological significance of the structures, since structure assumes its full significance only when correlated with function.

Each chapter ends with bibliographic references chosen for their excellence or because they contain many other important references.

Thirteenth edition revised throughout, with some extensive sections entirely rewritten. Includes the pertinent information resulting from recent marked advances in the field of histology—particularly in histochemistry, electron microscopy, and the elucidation of structural-functional relationships.

Thirteenth edition adds six new color plates, most of which contain several individual illustrations, and 88 halftone and line figures, some of which are replacements and others of which depict structures not previously shown. All the figures are from carefully-selected material and present typical structural features; all show cellular proportions and structural relations with fidelity.

Thirteenth edition succeeds even better than previous editions in bridging the gap between gross and microscopic anatomy.

793 pp. *442 figs. (31 in color)* *$9.00*

THE WILLIAMS & WILKINS COMPANY

Mt. Royal and Guilford Aves. Baltimore 2, Maryland

BRITISH MEDICAL BULLETIN

Volume 5, No. 4–5

Part I: NEW CURRENTS IN BIOCHEMISTRY

Cellular Function, Metabolism and Nutrition of Micro-organisms, Development and Applications of BAL, Human Biochemical Genetics, Viruses, Immunology, Proteins, and Amino-acids in Nutrition.

Part II: COMMENTARY, HISTORY, DOCUMENTATION

History of Chemotherapy, Iatrochemists, Chemotherapy in Typhus, etc. $3.00

Volume 9, No. 2

SOME ASPECTS ON ENZYME RESEARCH

Biochemical Lesions, Folic Acid in Metabolism, Plasma Alkaline Phosphatase in Disease, Adaptive Enzymes, Cytochrome, together with nine other articles. $2.75

Vol. 10, No. 3 **CHROMATOGRAPHY** $2.75

May be obtained in the U. S. A. from

OXFORD UNIVERSITY PRESS, INC.
114 Fifth Avenue, New York 11

Chemicals wanted by:

NATIONAL REGISTRY OF RARE CHEMICALS

Armour Research Foundation of the Illinois Institute of Technology
35 West 33rd Street, Chicago 16, Illinois

Solanidine	2,3-Dithiolsuccinic acid (*d* or *l*)
3,4-Dimethoxybenzoic acid	2,3-Dichlorosuccinic acid (*d* or *l*)
Ibogaine	N-Methyl-2-picolinic acid betaine
Avidin	L-Galactonic acid
Quinuclidine	Cellotriose
l-Threonic acid	Aloetic acid
Anatabine	Bismuth ethylcamphorate
N-Methyl anatabine	Coprostenol (allocholesterol)
Pseudocholinesterase	Campesterol
Mesobilirubinogen	*n*-Ethyl tetrahydroquinoline

Annual Reviews, Inc.

Vol. 6 Jan., 55 — **Psychology,** C. P. STONE, Q. MCNEMAR, *Editors*; 18 chapters, 517 pages.

Vol. 17 March, 55 — **Physiology,** V. E. HALL, F. A. FUHRMAN, A. C. GIESE, *Editors*; 21 chapters, 551 pages.

Vol. 6 May, 55 — **Medicine,** D. A. RYTAND, J. A. ANDERSON, *Editors*; 19 chapters, Annotated List of Reviews (523 references), 459 pages.

Vol. 6 June, 55 — **Plant Physiology,** D. I. ARNON, L. MACHLIS, *Editors*; 17 chapters, 505 pages.

Vol. 24 July, 55 — **Biochemistry,** J. MURRAY LUCK, H. S. LORING, G. MACKINNEY, *Editors*; 21 chapters, 805 pages.

Vol. 5 Sept., 54 — **Physical Chemistry,** G. K. ROLLEFSON, R. E. POWELL, *Editors*; 23 chapters, 540 pages.

Vol. 8 Oct., 54 — **Microbiology,** C. E. CLIFTON, S. RAFFEL, R. Y. STANIER, *Editors*; 22 chapters, 536 pages.

Vol. 4 Dec., 54 — **Nuclear Science,** J. G. BECKERLEY, M. D. KAMEN, L. I. SCHIFF, *Editors*; 17 chapters, 483 pages.

$7.00 postpaid (U.S.A.); $7.50 postpaid (elsewhere)

ANNUAL REVIEWS, INC., Stanford, California

CLEARING FACTOR, A HEPARIN-ACTIVATED LIPOPROTEIN LIPASE*

I. ISOLATION AND CHARACTERIZATION OF THE ENZYME FROM NORMAL RAT HEART

By EDWARD D. KORN

(From the Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, October 15, 1954)

The importance of plasma lipoproteins in normal and abnormal fat metabolism has stimulated interest in the heparin-induced lipemia clearing system first observed by Hahn (2). As a result of the efforts of several independent investigators (3, 4), it is now known that, upon the administration of heparin to animals of many different species, an enzyme appears in the blood which is able to "clear" lipemic serum *in vivo* or *in vitro*.

It has been difficult to evaluate the rôle of this "clearing factor" in normal fat metabolism since it normally exists in blood in extremely low concentrations, if at all (5). Certainly the clearing factor content of normal blood is insufficient to account for the rate of disappearance of alimentary lipemia. This is most readily evidenced by the fact that lipemic serum does not clear *in vitro*. Recent investigations of Bragdon and Havel (6) demonstrated that the administration of protamine and other antiheparinoid agents to fasting rats increases the serum lipide and lipoprotein concentration and, when injected simultaneously with lipoproteins, delays their removal. These results suggest that a heparinoid-requiring system is involved in normal fat metabolism.

The work to be reported in this paper demonstrates the presence in normal rat heart of an enzyme identical with the clearing factor which appears in blood upon heparin administration. In this and the accompanying paper (7), evidence is presented which supports the view that clearing factor is a heparin-activated lipoprotein lipase which may play a major rôle in lipide metabolism.

Materials and Methods

Sodium heparin, 126 units per mg., was generously supplied by The Upjohn Company, protamine sulfate was a product of the Nutritional Biochemicals Corporation, and the commercial pancreatic lipase was obtained from the Fisher Scientific Company. The coconut oil emulsion was sup-

* A preliminary report of some of the data presented in this and the accompanying paper has been published (1).

XUM

plied by the Abbott Laboratories. It had the following percentage composition (weight per volume): coconut oil 15, glucose 5, Tween 60 0.5, phenylmercuric nitrate 0.002, and tocopherol 0.01. It was diluted to a coconut oil concentration of 1.5 per cent before use. The albumin used was bovine serum albumin obtained from the Armour Laboratories; the chylomicrons were obtained from pooled blood from the American Red Cross. The material of low density, which separated on standing in the refrigerator, was washed several times with 0.15 M NaCl by centrifugation and used after dilution with water to approximately 1.5 per cent neutral fat content. Serum was whole normal human serum.

Acetone powders were prepared by homogenizing the tissues in approximately 20 volumes of acetone (−20°) in a Waring blendor and filtering the mixture rapidly at room temperature. The filter cake was blended again with acetone and the final powder stored in a vacuum desiccator at −20°. Aortas were homogenized briefly in a Potter-Elvehjem glass homogenizer before the acetone treatment.

In all the experiments, unless otherwise stated, the incubation vessel contained 0.2 ml. of 10 per cent albumin (pH 8.5), 0.02 ml. of 1 M $CaCl_2$, 0.1 ml. of the chylomicron suspension, and 0.28 ml. of 0.25 M NH_4Cl-NH_3 buffer (pH 8.5) in a total volume of 1 ml. Other additions were as indicated. Duplicate 0.05 ml. aliquots of the incubation mixture were removed at 0, 30, 60, and 120 minutes, in all cases, and added directly to 0.1 ml. of cold 1 N H_2SO_4 in a 12 ml. graduated centrifuge tube. Glycerol was then determined by a modified procedure of Lambert and Neish (8). This method is highly reproducible and enables one routinely to determine from 0.005 to 0.1 μmole of glycerol. The contents of the centrifuge tube were warmed to room temperature and 0.1 ml. of 0.05 M sodium periodate was added. After 5 minutes, 0.1 ml. of 0.5 M sodium arsenite was added to reduce the excess periodate. After an additional 10 minutes, 9 ml. of chromotropic acid reagent (a formaldehyde reagent) were added, and the reaction tubes placed in a covered boiling water bath for 30 minutes. The cooled tubes were adjusted to a volume of 10 ml. with water and the optical density read at 570 mμ in a Coleman junior spectrophotometer. By this procedure 2 moles of formaldehyde are formed from 1 mole of glycerol, and 1 mole of formaldehyde from 1 mole of α-monoglyceride. All the values were calculated in terms of glycerol, although an undetermined percentage of the formaldehyde was actually derived from α-monoglycerides. All the values reported are the averages of two determinations, and in all instances the 30, 60, and 120 minute values were proportional to the incubation times. Where only one value is reported, it is the one obtained after an incubation period of 120 minutes.

Results

Production of Glycerol during Incubation of Clearing Factor with Coconut Oil[1]—Several investigators (4, 9) have observed that fatty acids are released during the action of plasma clearing factor on natural and synthetic fat emulsions. In the experiment described in Fig. 1, the rate of clearing of a coconut oil emulsion was compared to the rate of formation of glycerol. The enzyme employed was an aliquot of whole, postheparin dog plasma.

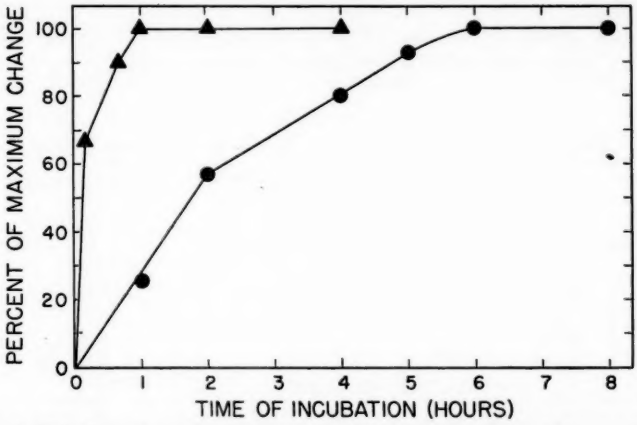


FIG. 1. Relative rates of clearing (▲) and glycerol production (●) upon incubation of a coconut oil emulsion with clearing factor. The incubation vessel contained 0.2 ml. of 0.75 per cent coconut oil, 0.4 ml. of postheparin dog plasma, and 1.4 ml. of saline-phosphate buffer. In a control vessel, normal dog serum was used instead of postheparin plasma.

It is evident that glycerol was produced, although the rate of production of glycerol was much slower than the rate of clearing. In contrast to this, the rate of fatty acid production, in similar experiments,[2] was virtually identical with the rate of clearing. This is not unexpected, since one-third of the fatty acids could be released before the formation of a compound which would yield formaldehyde upon periodate oxidation.

[1] It is understood that α-monoglycerides as well as glycerol are included in the assay. If the incubation is continued for a sufficiently long period, all the substrate can be hydrolyzed completely to glycerol.

[2] In these experiments, which were performed in collaboration with Dr. R. S. Gordon, postheparin dog plasma was incubated with coconut oil in the presence of excess albumin. Fatty acid, glycerol, and turbidity determinations were made concomitantly.

The production of compounds (glycerol, α-monoglycerides) which yield formaldehyde upon oxidation with periodate provides an extremely convenient means for assaying the clearing factor. It is much more accurate than, and not subject to the numerous difficulties of, the turbidity method employed in previous studies of this enzyme.

Extraction of Clearing Factor from Rat Heart Acetone Powder—Anfinsen *et al.* (5) reported that clearing factor is obtained upon the incubation of heart and lung preparations with heparin and normal plasma. This was interpreted as indicating the existence of a "tissue factor" which converted a "plasma precursor substance" into clearing factor in the presence of heparin. These observations have been reinvestigated.

TABLE I

Extraction of Lipoprotein Lipase from Rat Heart Acetone Powder

Experiment No.	Extracting solvent	Glycerol production		
		30 min.	60 min.	120 min.
		μmole	μmole	μmole
1	Serum		0.21	0.43
2	Saline-phosphate	0	0	0
3	NH_3	0.19	0.39	0.61
4	Serum (after NH_3)	0.03	0.05	0.11
5	" saline-phosphate, or NH_3*	0	0	0

All the vessels contained 0.2 ml. of the solution to be assayed in addition to the other components, as described under "Methods." All of the incubation vessels contained an equal amount of serum. For other experimental details, see the text.

* In this experiment the three solvents were each assayed separately. No acetone powder was present at any time.

50 mg. of rat heart acetone powder were extracted for 30 minutes at 0° with 1 ml. of normal human serum, or saline-phosphate buffer (1 part of 0.15 M NaCl:1 part of phosphate buffer (pH 7.4, $\Gamma/2 = 0.1$)), or 0.025 N ammonia. The insoluble residues were removed by centrifugation (25,000 × *g*) and the supernatant fluids incubated at 38° for 30 minutes with addition of an equal volume of serum to those samples which did not already contain it. In one instance, Experiment 4, the acetone powder was extracted with 0.025 N ammonia, the supernatant fluid discarded, and the residue washed with saline-phosphate and extracted again with serum, which was then assayed for activity.

As shown in Table I, both the serum and ammonia extracts contained an active component, while the saline-phosphate extract was inactive. The serum extract of the acetone powder which had been previously extracted

with ammonia had relatively little lipase activity, and none of the three extracting solvents alone (Experiment 5) had any activity.

The results tabulated in Table II demonstrate that the ammonia extract had lipolytic activity in the complete absence of serum and that preincubation of the ammonia extract with serum, even in the presence of heparin, had no stimulatory effect. Thus the rat heart acetone powder contains a lipase which will be shown to be identical to clearing factor, and the serum serves only as a solvent which can be completely replaced by ammonia.

The rate of glycerol production was directly proportional to the amount of enzyme added (Fig. 2) and to the time of incubation (see Table II, for example). The enzyme is non-dialyzable and stable to dialysis for 24 hours against distilled water. It is completely inactivated when heated

TABLE II

Non-Essentiality of Serum for Preparation of Lipoprotein Lipase

Experiment No.	Serum added to assay vessel	Glycerol production		
		30 min.	60 min.	120 min.
	ml.	*μmole*	*μmole*	*μmole*
1	0	0.18	0.38	0.65
2	0.2	0.13	0.26	0.49
3	0.2*	0.12	0.24	0.49

All the vessels contained 0.2 ml. of the ammonia extract of rat heart acetone powder and 10 γ of heparin in addition to the usual components.

* In this experiment the ammonia extract was preincubated with the serum and heparin for 30 minutes at 38° before its lipolytic activity was assayed.

in a boiling water bath for 5 minutes. The ammonia extract of the rat heart acetone powder will be referred to as lipoprotein lipase, since this denotes both its specificity and the type of reaction which it catalyzes, as will be more fully demonstrated in the accompanying paper (7). Unless otherwise indicated, it was always prepared by extracting 50 mg. of rat heart acetone powder with 1 ml. of 0.025 N ammonia for 30 minutes at 0°.

Effect of Heparin on Lipoprotein Lipase—There has been no evidence that a heparinoid is necessary for the enzymatic action of postheparin plasma clearing factor. Heparin added *in vitro* to such a clearing factor preparation inhibits its activity (4). The addition of small amounts of heparin to the ammonia extract of rat heart acetone powder stimulated the lipase activity approximately 2-fold (Table III). Relatively large amounts of heparin had an inhibitory effect. In some instances 3- and 4-fold stimulations have been obtained. Mactin-A (a sulfated polysaccharide from clams) and polymetaphosphate, both of which can produce plasma

XUM

clearing factor *in vivo* (10), also stimulate lipoprotein lipase, but to a lesser extent than heparin.

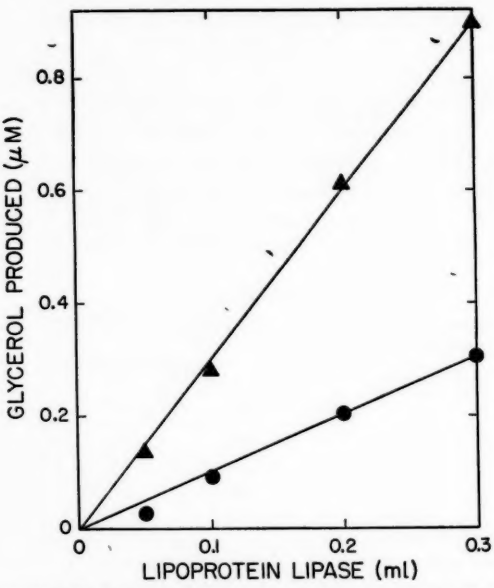


FIG. 2. Proportionality between lipoprotein lipase concentration and glycerol production (●, 30 minutes; ▲, 120 minutes).

TABLE III

Effect of Heparin on Lipoprotein Lipase

Experiment No.	Heparin	Glycerol production
	γ	*μmole*
1	0	0.29
2	0.1	0.51
3	0.3	0.57
4	5.0	0.66
5	100.0	0.27

All the vessels contained 0.1 ml. of lipoprotein lipase. The other conditions are as described under "Methods."

Inhibition of Lipoprotein Lipase by Salt and Protamine—In addition to the effect of heparin, the only distinguishing characteristic of postheparin plasma clearing factor is its pronounced sensitivity to relatively low con-

centrations of salt and protamine (4). The inhibitory effects of these compounds on the rat heart preparation are shown in Table IV.

The inhibition by protamine is presumably due to the presence of enzyme-bound heparin, which is apparently an essential component of the system. Recent experiments, in collaboration with Mr. Thomas W. Quigley, Jr., on lipoprotein lipase extracted from calf heart, have demonstrated that heparin can reverse this inhibition. The inhibition by salt is not reversible by heparin at any concentration nor by the addition of whole serum or a boiled extract of lipoprotein lipase. On the assumption that the active enzyme contains bound heparin, an attempt was made to in-

TABLE IV

Effect of Salt and Protamine on Lipoprotein Lipase

Experiment No.	Pretreatment of enzyme	Lipase	Heparin	Glycerol production
		ml.	*γ*	*μmole*
1	None	0.1		0.40
2	"	0.1	10	0.65
3	NaCl, 1 M	0.2		0
4	" 1 "	0.2	10	0
5	KCl, 1 M	0.2		0
6	" 1 "	0.2	10	0
7	Protamine, 5×10^{-5} M	0.1		0.10
8	" 1×10^{-5} "	0.1		0.27
9	" 1×10^{-6} "	0.1		0.33

The other additions are as under "Methods." Aliquots of lipoprotein lipase were incubated with NaCl, KCl, or protamine in the concentrations indicated for 30 minutes at 0°. All the samples were dialyzed overnight against distilled water and then assayed.

activate it by treatment with Dowex 1 Cl^- and activated carbon. Both attempts were unsuccessful.

Effects of Heparin, Salt, and Protamine on Pancreatic Lipase—The effects of heparin, salt, and protamine on lipoprotein lipase are quite unique. They serve to distinguish it from other lipases which will also hydrolyze (although not as well) chylomicrons. Pancreatic lipase, for example, was neither activated by heparin nor inactivated by salt or protamine under conditions identical with those used to demonstrate the effects of these compounds on lipoprotein lipase.

Inhibition of Lipoprotein Lipase by Pyrophosphate—The data tabulated in Table V demonstrate that sodium pyrophosphate completely inhibited lipoprotein lipase when present in the assay vessel at a concentration of 10 μmoles per ml. At levels of 5 μmoles per ml. the pyrophosphate inhibi-

XUM

tion was not always complete but was still considerable, and could then be reversed by 5 γ of heparin. This inhibition could also be overcome by polymetaphosphate.

TABLE V

Inhibition of Lipoprotein Lipase by Pyrophosphate

Experiment No.	Sodium pyrophosphate	Heparin	Glycerol production
	μmoles	γ	*μmole*
1			0.46
2		5	0.68
3	1		0.49
4	1	5	0.63
5	5		0.14
6	5	5	0.57
7	5		0
8	5	5	0.51
9	10		0
10	10	5	0

All the vessels contained 0.1 ml. of lipoprotein lipase.

TABLE VI

Inhibition of Lipoprotein Lipase by Phosphate-Containing Compounds

Experiment No.	Inhibitor*		Heparin	Glycerol production
		μmoles	γ	*μmole*
1				0.34
2			5	0.55
3	P	50		0.14
4	"	50	5	0.42
5	ATP	20		0
6	"	10		0.10
7	"	10	5	0.40
8	UTP	10		0.07
9	"	10	5	0.44
10	ITP	20		0.05
11	AMP	20		0.05
12	"	10		0.24
13	"	10	5	0.44

All the vessels contained 0.1 ml. of lipoprotein lipase.

* P, orthophosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; ITP, inosine triphosphate; AMP, adenylic acid.

Other phosphate derivatives were also assayed for their ability to inhibit lipoprotein lipase (Table VI). Inorganic phosphate, adenosine triphosphate, uridine triphosphate, inosine triphosphate, and adenylic acid all

inhibited, although at somewhat higher concentrations than was necessary with the pyrophosphate. Heparin could reactivate the enzyme in all cases.

This inhibition is due to a direct effect of the phosphate compound on lipoprotein lipase itself. Pyrophosphate had no inhibitory effect when present in the assay vessel at a concentration of 0.5 μmole per ml. However, preincubation of the enzyme with pyrophosphate at a concentration of 5 μmoles per ml. caused definite inhibition even though the inhibitor was diluted to the non-effective concentration in the assay vessel (Table VII).

TABLE VII

Effect of Preincubation of Lipoprotein Lipase with Pyrophosphate

Experiment No.	Sodium pyrophosphate	Heparin	Glycerol production
	μmoles	γ	*μmole*
1			0.49
2		5	0.57
3	0.5		0.50
4	0.5	5	0.58
5	5.0		0.19
6	5.0	5	0.50
7	0.5*		0.27
8	0.5*	5	0.43

All the vessels contained 0.1 ml. of lipoprotein lipase.

* In these two experiments, 0.5 μmole of pyrophosphate was preincubated with 0.1 ml. of lipoprotein lipase for 30 minutes at room temperature. In the absence of pyrophosphate, there was no decrease in enzymatic activity during a preincubation period at room temperature.

These results are most readily explained by the assumption that lipoprotein lipase, as isolated from normal rat hearts, contains enzyme-bound heparin (or heparinoid) which may be competitively replaced by polyvalent anions which possess no cofactor activity.

Requirement for Fatty Acid Acceptor—Recent experiments by Gordon *et al.* (11) have demonstrated that serum albumin stimulates the clearing of coconut oil emulsions by postheparin plasma clearing factor by virtue of its ability to remove from solution the fatty acids produced during the reaction. Other investigators (12) have postulated a special, and more obscure, rôle for albumin. It can be shown, however, that there is no specific requirement for albumin for the hydrolysis of chylomicrons by lipoprotein lipase (Fig. 3). The chylomicrons used as substrate in this experiment were washed completely free of albumin. Calcium ions were at least as good a fatty acid acceptor as albumin.

In the experiments reported in this and the accompanying paper, both

albumin and calcium ions were present, although calcium ions alone were sufficient to insure maximal activity. The albumin served to maintain an elevated protein concentration and thus stabilize the enzyme during the longer incubation periods.

Distribution of Lipoprotein Lipase—Lipoprotein lipase is present in beef and pig hearts, and experiments are currently in progress to purify the beef heart enzyme. Acetone powders of various rat tissues were surveyed for activity. Ammonia extracts of liver, kidney, spleen, and lung acetone

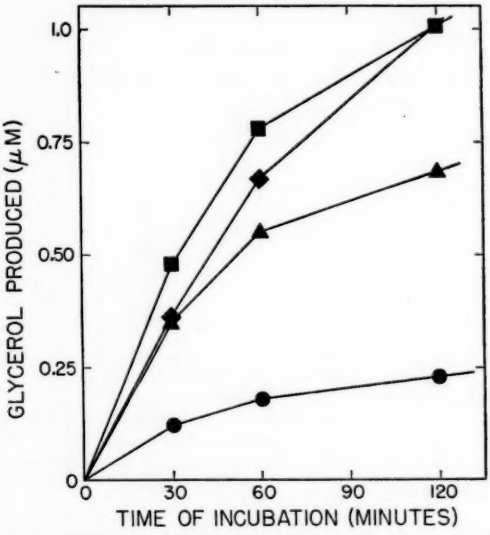


FIG. 3. Requirement for a fatty acid acceptor. All of the vessels contained 0.2 ml. of lipoprotein lipase and 10 γ of heparin. The fatty acid acceptors were as follows: ●, none; ▲, 0.2 ml. of 10 per cent albumin; ◆, 0.02 ml. of 1 M $CaCl_2$; ■, 0.02 ml. of 1 M $CaCl_2$ + 0.2 ml. of 10 per cent albumin.

powders showed some lipolytic activity with chylomicrons as substrate but much less than the heart preparation (Table VIII). Skeletal muscle preparations were completely inactive and aorta preparations virtually so. Perhaps the best indication of lipoprotein lipase content is the decrease in activity after preincubation with 1 M NaCl. By this criterion all the lipolytic activity of aorta and spleen, but less than half that of liver, would be due to lipoprotein lipase. None of the lipolytic activity of lung would appear to be due to lipoprotein lipase. Heparin did not stimulate the activity of any of the preparations so that the lipoprotein lipase, if present, must be saturated with heparin. As will be described in detail in the accompanying paper (7), lipoprotein lipase will not hydrolyze coconut oil in

the absence of serum. The activity of the liver, spleen, kidney, and aorta extracts with coconut oil as substrate indicates the presence of some other lipase in these tissues. It is possible that these tissues contain more lipoprotein lipase than indicated here if, for some reason, the enzyme is less

TABLE VIII

Distribution of Lipoprotein Lipase in Rat

Tissue	Amount extracted	Substrate	Addition	Glycerol producton
	mg. per ml.			*μmole*
Liver	50	Chylomicrons	None	0.12
		"	Heparin	0.11
		"	NaCl	0.07
		"	Protamine	0.08
		Coconut oil	None	0.29
Kidney	50	Chylomicrons	"	0.17
		"	Heparin	0.21
		"	NaCl	0.05
		"	Protamine	0.16
		Coconut oil	None	0.17
Spleen	50	Chylomicrons	"	0.07
		"	Heparin	0.08
		"	NaCl	0
		"	Protamine	0.05
		Coconut oil	None	0.12
Aorta	240	Chylomicrons	"	0.14
		"	Heparin	0.12
		"	NaCl	0
		"	Protamine	0.08
		Coconut oil	None	0.17
Lung	50	Chylomicrons	"	0.08
		"	Heparin	0.08
		"	NaCl	0.08
		"	Protamine	0.08
Skeletal muscle	50	"	None	0
		"	Heparin	0

Where indicated, the enzyme was preincubated at 0° for 30 minutes with 1 M NaCl or 5×10^{-5} M protamine. 10 γ of heparin were added at the time of assay when heparin was present.

stable or less readily extractable than the heart enzyme. It must be concluded, however, that the presence of appreciable quantities of lipoprotein lipase in any tissue other than heart remains to be demonstrated.[3]

[3] Further experiments, in collaboration with Mr. Thomas W. Quigley, have demonstrated that relatively large quantities of lipoprotein lipase can be extracted from rat and rabbit adipose tissue. Adipose tissue appears to be the major source of the enzyme, which emphasizes its importance in fat transport.

XUM

Lipoprotein Lipase Content of Rat Hearts after Heparin Administration—Since a large part of the lipoprotein lipase of the rat is apparently localized in the heart, it was thought that the administration of heparin might deplete the heart of the enzyme or at least saturate it with heparin. An amount of heparin was used in these experiments which would produce

TABLE IX

Effect of Heparin in Vivo on Clearing Factor Content of Rat Heart

Pretreatment	Experiment No.	Heparin	Glycerol production
			μmole
Saline	1	−	0.37
		+	0.46
	2	−	0.21
		+	0.43
	3	−	0.40
		+	0.54
	4	−	0.46
		+	0.52
Average		−	0.36
		+	0.49
Heparin	5	−	0.16
		+	0.30
	6	−	0.15
		+	0.24
	7	−	0.28
		+	0.46
	8	−	0.29
		+	0.42
Average		−	0.22
		+	0.35

All of the assay vessels contained 0.1 ml. of lipoprotein lipase, and 10 γ of heparin were added where indicated above. For further experimental details, see the text.

approximately as much lipoprotein lipase in the blood of one rat as is normally found in the acetone powder of one heart.

Heparin (1 mg. per kilo) was injected into the tail vein of eight 200 gm. rats and eight control rats received an equal volume of physiological saline (0.5 ml.). The animals were decapitated 15 minutes after the injection (the time required for the lipoprotein lipase activity of the blood to reach a maximal level) and acetone powders of the hearts prepared. Two hearts were pooled in order to have sufficient material. Therefore, four control and four experimental values were obtained. In all cases, 50 mg. of powder

were extracted with 1 ml. of 0.025 N ammonia for 30 minutes at 0°, the mixture was centrifuged, and the clear supernatant fluid assayed.

Although the series is not sufficiently great to permit a definite conclusion, there was an apparent tendency for the hearts of the heparin-treated animals to have a lower lipoprotein lipase content than the controls (Table IX). A major portion of the original activity still remained, however, and the loss does not appear to be sufficient to account for the enzyme level in the blood. This might be explained by the contribution to the blood from other sources, or by an increased synthesis of enzyme protein in response to the elevated heparin concentration. It is obvious that the lipoprotein lipase had not been saturated with heparin, since its activity was stimulated by heparin added *in vitro*.

DISCUSSION

The experiments described in this paper definitely establish the presence in normal rat heart of an enzyme with all the known properties of postheparin plasma clearing factor. This removes the major obstacle in the attempt to relate the phenomena which occur in postheparin plasma to normal lipide metabolism. These phenomena do occur in the absence of injected heparin but at the cellular level. The administration of heparin, therefore, appears to cause an overflow of the lipase into the blood, where it does not exist normally in significant quantities.

SUMMARY

Clearing factor, the enzyme present in postheparin plasma, can be extracted from acetone powders of normal rat hearts. This enzyme catalyzes the hydrolysis of the neutral fat of chylomicrons. It is activated by heparin and inhibited by salt and protamine. A fatty acid acceptor is also required. The distribution of the enzyme in other tissues and the effect of heparin administration on the concentration of the enzyme in heart have been investigated.

The author wishes to thank Dr. Christian B. Anfinsen for his continued interest in this problem.

BIBLIOGRAPHY

1. Korn, E. D., *Science*, **120**, 399 (1954).
2. Hahn, P. F., *Science*, **98**, 19 (1943).
3. Anderson, N. G., and Fawcett, B., *Proc. Soc. Exp. Biol. and Med.*, **74**, 768 (1950).
4. Brown, R. K., Boyle, E., and Anfinsen, C. B., *J. Biol. Chem.*, **204**, 423 (1953).
5. Anfinsen, C. B., Boyle, E., and Brown, R. K., *Science*, **115**, 583 (1952).
6. Bragdon, J. H., and Havel, R. J., *Am. J. Physiol.*, **177**, 128 (1954).
7. Korn, E. D., *J. Biol. Chem.*, **215**, 15 (1955).

8. Lambert, M., and Neish, A. C., *Canad. J. Res.*, **28,** 83 (1950).
9. Shore, B., Nichols, A. V., and Freedman, N. K., *Proc. Soc. Exp. Biol. and Med.*, **83,** 216 (1953).
10. Havel, R. J., and Bragdon, J. H., *Circulation*, **10,** 591 (1954).
11. Gordon, R. S., Boyle, E., Brown, R. K., Cherkes, A., and Anfinsen, C. B., *Proc. Soc. Exp. Biol. and Med.*, **84,** 168 (1953).
12. Nikkilä, E. A., and Haahti, E., *Acta chem. Scand.*, **8,** 363 (1954).

CLEARING FACTOR, A HEPARIN-ACTIVATED LIPOPROTEIN LIPASE

II. SUBSTRATE SPECIFICITY AND ACTIVATION OF COCONUT OIL

By EDWARD D. KORN

(From the Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, October 15, 1954)

In the first paper of this series (1), evidence was presented which demonstrates that the clearing factor which appears in postheparin plasma is present in the hearts of normal, non-heparinized rats. This strongly supports the view that the enzyme functions in normal fat metabolism. In this paper, the substrate specificity of lipoprotein lipase is defined, the conversion of coconut oil into an available substrate described, and the possible rôle of the enzyme in fat transport discussed.

Materials and Methods

Substrates—The chylomicron suspension and coconut oil emulsion were prepared as described previously (1). Both preparations had a neutral fat concentration of approximately 1.5 per cent. Two different samples of soluble lipoproteins were used. One was prepared from the serum of a normal male 3 hours after the ingestion of a fatty meal which consisted primarily of 200 gm. of oleomargarine. The chylomicrons were removed by centrifugation at a relatively low speed and the remainder of the serum centrifuged for 12 hours at a centrifugal force of 105,000 $\times$ g. The tube was sliced and the top layer collected. This solution contained the β-lipoproteins of low density (S_f 20 and above by the nomenclature of Gofman *et al.* (2)). The preparation was slightly opalescent but was definitely free of chylomicrons. Analysis revealed the following composition in mg. per cent: total cholesterol 26, free cholesterol 14, phospholipide 150, and neutral fat 704. This preparation will be referred to as soluble lipoproteins I.

The second sample of soluble lipoproteins was prepared by the identical procedure from the serum of a male with idiopathic hyperlipemia. This solution was completely clear and had the following composition in mg. per cent: total cholesterol 106, free cholesterol 33, phospholipide 203, and neutral fat 347. This preparation will be referred to as soluble lipoproteins II.[1] It was indistinguishable from soluble lipoproteins I with respect to lipoprotein lipase action.

[1] The author is grateful to Dr. R. J. Havel and Dr. E. Boyle for the preparation of the soluble lipoproteins and to Dr. J. H. Bragdon for their analysis.

XUM

Enzymes—Lipoprotein lipase was prepared by extracting rat heart acetone powder with 0.025 N ammonia (50 mg. per ml.) for 30 minutes at 0°. The clear supernatant solution obtained upon centrifugation was used. Pancreatic lipase was prepared by extracting commercial steapsin (Fisher Scientific Company) with 0.025 N ammonia (1 mg. per ml.).

Assay Procedure—The substrate, enzyme, and other components were mixed at 0° and then incubated at 37°. All the vessels contained 0.2 ml. of 10 per cent albumin (pH 8.5), 0.02 ml. of 1 M $CaCl_2$, 10 γ of heparin, and 0.28 ml. of 0.25 M NH_4Cl-NH_3 buffer (pH 8.5) in a total volume of 1 ml. Other additions or any special techniques are described in the text. Duplicate aliquots of 0.05 ml. were removed at 0, 15, 30, and 60 minutes, and added immediately to 0.1 ml. of cold 1 N H_2SO_4. Glycerol was then determined by the method described in the previous paper (1). Where only one value is reported, it is that obtained after incubation for 60 minutes.

Results

Substrate Specificity—Lipoprotein lipase catalyzes the hydrolysis of the neutral fat of lipoproteins only (Fig. 1). Chylomicrons and soluble lipoproteins appear to be hydrolyzed equally well. There is little, if any, hydrolysis of coconut oil, the values reported here being the highest obtained in numerous assays. The repeated observation that postheparin plasma clearing factor can clear the turbidity of synthetic triglyceride emulsions (3, 4) is in apparent conflict with this specificity of rat heart lipoprotein lipase and would seem to indicate a major difference between the two enzymes. This apparent difference is explained by the observation that coconut oil becomes available as a substrate for lipoprotein lipase upon the addition of as little as 0.005 ml. of whole normal serum to the incubation vessel (Fig. 1).

In marked contrast to lipoprotein lipase, pancreatic lipase catalyzes the hydrolysis of coconut oil at a more rapid initial rate than chylomicrons which, in turn, are hydrolyzed more rapidly than soluble lipoproteins (Fig. 2).

Activation of Coconut Oil by Whole, Normal, Human Serum—In view of the pronounced specificity of lipoprotein lipase, the simplest interpretation of the activation of coconut oil by serum is that a lipide-protein complex is formed between the triglyceride and a serum protein. In Table I are tabulated the results of a series of experiments in which coconut oil was preincubated for 30 minutes at 37° with different components of the complete system.[2] At the end of the preincubation period, all of the vessels were chilled to 0° and the remainder of the constituents added so that

[2] The complete system in these experiments contained 0.2 ml. of whole, normal, human serum in addition to the components listed under "Methods."

during the subsequent incubation period all of the vessels were identical. The only exceptions were Experiment 1, in which no serum was present at any time, and Experiment 4, in which the preincubation was carried out at 0°.

The difference in glycerol production between Experiments 1 and 2 demonstrates the effect of serum on the hydrolysis of coconut oil by lipoprotein lipase. There was an even greater activation when the serum was

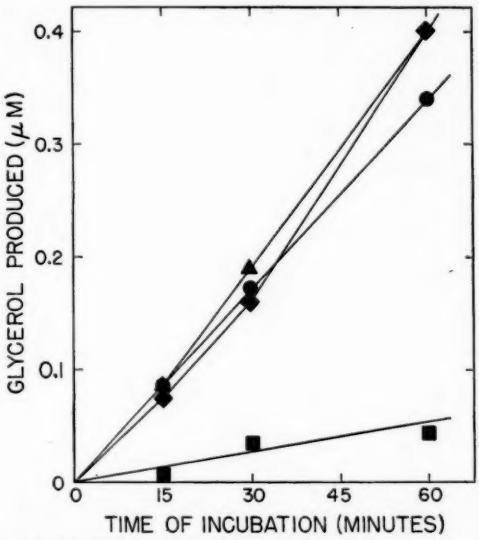


FIG. 1. Substrate specificity of lipoprotein lipase. All the vessels contained the components listed under "Methods," 0.1 ml. of lipoprotein lipase, and substrate as follows: ▲, 0.1 ml. of chylomicrons; ●, 0.2 ml. of soluble lipoproteins I or 0.4 ml. of soluble lipoproteins II; ■, 0.1 ml. of coconut oil; ◆, 0.1 ml. of coconut oil + 0.005 ml. of serum.

preincubated with the coconut oil (Experiment 3). The preincubation of serum and coconut oil was not nearly as effective when carried out at 0° (Experiment 4).

In contrast to the marked effect of serum, preincubation with any of the other components of the complete medium, in the presence or absence of serum, had no influence at all on the rate of hydrolysis of the coconut oil. The serum could not be replaced by either bile salts or synthetic detergents. The lowered activity in Experiment 7 was due to the partial inactivation of the enzyme during the preincubation period resulting from its instability in dilute protein solutions.

The serum used in these experiments was relatively low in activity as

compared to that used in the other experiments reported in this paper. For this reason, it was possible to demonstrate the increased activation during a preincubation period and thus determine the essential components. With the more potent sera, complete activation occurs immediately upon the addition of serum to the assay vessel, and it is impossible to separate the activation phenomenon from the enzymatic hydrolysis of the activated coconut oil.

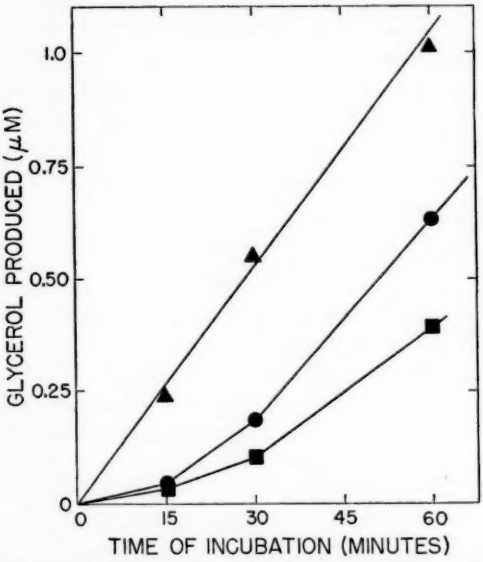


FIG. 2. Substrate specificity of pancreatic lipase. In addition to the usual components, all the vessels contained 0.3 ml. of pancreatic lipase and substrates as follows: ▲, 0.1 ml. of coconut oil; ●, 0.1 ml. of chylomicrons; ■, 0.2 ml. of soluble lipoproteins I or 0.4 ml. of soluble lipoproteins II.

The rate of hydrolysis of activated coconut oil by lipoprotein lipase is proportional to the amount of serum with which the triglyceride is preincubated (Fig. 3). The direct proportionality between the concentration of serum and the amount of hydrolysis which resulted in this experiment was not always obtained, although increasing the concentration of serum always resulted in more hydrolysis.

The serum retained complete activity after dialysis for 24 hours against large volumes of 0.15 M NaCl. However, only 60 per cent of the activity was lost when the serum was heated at 100° for 20 minutes. This rela-

TABLE I

Essential Components for Activation of Coconut Oil

Experiment No.	Addition during preincubation	Glycerol produced		
		15 min.	30 min.	60 min.
		μmole	*μmole*	*μmole*
1	None*	0	0	0
2	"	0.04	0.16	0.48
3	Serum	0.28	0.6	0.99
4	" (0°)	0.10	0.31	0.67
5	Albumin, $CaCl_2$, heparin	0.09	0.19	0.49
6	" " " serum	0.23	0.49	0.90
7	Lipoprotein lipase	0.03	0.12	0.36

The experimental procedure is described in the text.

* In Experiment 1, serum was not added after the preincubation period.

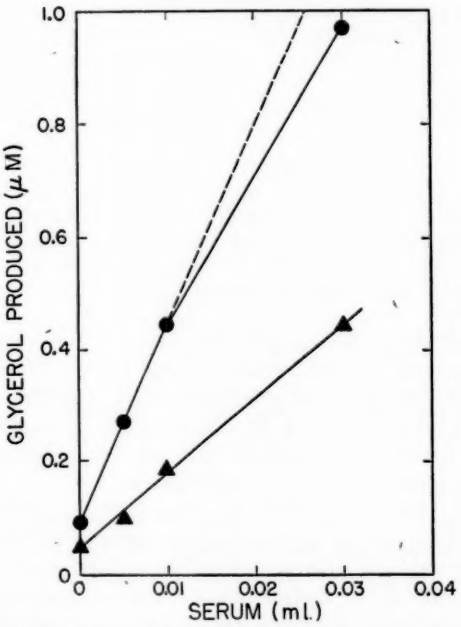


FIG. 3. Activation of coconut oil as a function of serum concentration. In this experiment, the serum, coconut oil, and sufficient water to adjust all the vessels to the same volume were preincubated for 30 minutes at 37°. All the vessels contained 0.2 ml. of lipoprotein lipase. ▲, incubated 30 minutes; ●, incubated 60 minutes.

tively high degree of heat stability might be explained if soluble, denatured proteins were still able to activate coconut oil to some extent.

Non-Effect of Serum on Hydrolysis of Chylomicrons—If the activation of coconut oil by serum were due to something other than the formation of a lipide-protein complex, it would seem likely that serum might also stimulate the hydrolysis of chylomicrons. However, the preincubation of chylomicrons with serum had no effect on the rate of their hydrolysis upon subsequent incubation with lipoprotein lipase. This would be the expected result if the activation of coconut oil were due to the postulated mechanism since chylomicrons are already lipoproteins.

Effect of Heparin, Salt, and Protamine on Hydrolysis of Activated Coconut Oil by Lipoprotein Lipase—It may readily be demonstrated that the

TABLE II

Effect of Heparin, Salt, and Protamine on Hydrolysis of Activated Coconut Oil by Lipoprotein Lipase

Experiment No.	Pretreatment of enzyme	Heparin	Glycerol produced
		μmoles	*μmole*
1	None	0	0.17
2	"	10	0.58
3	NaCl	10	0.00
4	Protamine	10	0.10

In Experiments 3 and 4, the lipoprotein lipase was preincubated for 30 minutes at 0° with 1 M NaCl and 5×10^{-5} M protamine, respectively. Heparin, when present, was added with the other components. The substrate was coconut oil preincubated with 0.05 ml. of serum for 30 minutes at 37°.

hydrolysis of activated coconut oil is due to lipoprotein lipase rather than some other component of the acetone powder extract. The reaction was inhibited when the enzyme was preincubated with 1 M NaCl or 5×10^{-5} M protamine, and was stimulated by the addition of heparin (Table II). The degree of activation by heparin was usually greater with activated coconut oil as substrate than with chylomicrons.

Preparation of Activated Coconut Oil—The foregoing data indicate that there is a reaction between a serum protein and coconut oil to form what might be considered, at least in some respects, a "chylomicron." It should be possible, therefore, to prepare such a lipide-protein complex which would then be an active substrate for lipoprotein lipase in the complete absence of serum.

4 ml. of 1.5 per cent coconut oil, 4 ml. of serum, and 4 ml. of water were incubated together for 30 minutes at 37°. The mixture was then centrifuged and the oil layer (2 ml.) collected. This oil layer was re-

peatedly suspended in water and concentrated by centrifugation until any serum component which was not specifically adsorbed onto the triglyceride would have been diluted at least 3000-fold. This oil (0.1 ml.) was used as substrate in the experiments described in Figs. 4 and 5. There could not have been more than 0.00003 ml. of the original serum present in the in-

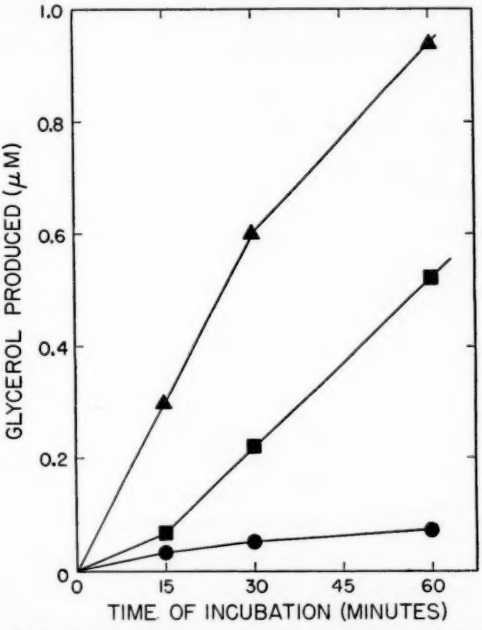


FIG. 4. Hydrolysis of activated coconut oil by lipoprotein lipase in the complete absence of serum. The substrates were ●, coconut oil; ■, coconut oil + serum; ▲, activated coconut oil with or without added serum. All the vessels contained 0.2 ml. of lipoprotein lipase.

cubation vessel, an amount well below the minimal volume necessary for activation of coconut oil (the same serum was used as in Fig. 1). Further, a subsequent washing of this activated oil with 20 volumes of water did not decrease its activity.

It is clear from Fig. 4 that a complex had been formed during the interaction of serum and coconut oil which was readily hydrolyzable by lipoprotein lipase in the complete absence of serum. It was even hydrolyzed at a much more rapid rate than coconut oil which was preincubated with serum and used as substrate in the presence of the serum. Preincubation

of the activated coconut oil with serum had no effect on the rate of its hydrolysis.

Chylomicrons are hydrolyzed more slowly than coconut oil by pancreatic lipase (Fig. 2). If the activated coconut oil is, in fact, a lipide-protein complex, it should be a poorer substrate than the original coconut oil for pancreatic lipase. This was found to be the case (Fig. 5). This supports the view that the action of serum is not concerned with the partial hydroly-

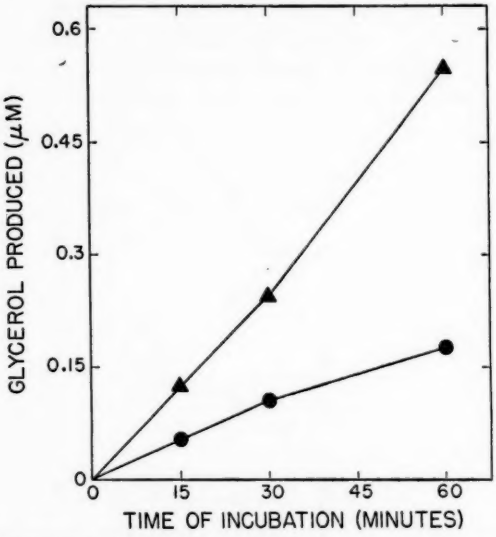


FIG. 5. Decreased rate of hydrolysis of activated coconut oil by pancreatic lipase. The components of the incubation medium were the same as described under "Materials and methods," except that 0.3 ml. of pancreatic lipase was the enzyme. The substrates were ●, activated coconut oil; ▲, coconut oil.

sis or better emulsification of the coconut oil since these or similar changes would make it a better substrate for pancreatic lipase as well as for lipoprotein lipase. In view of the known specificities of the two enzymes, the conversion of the coconut oil into a lipide-protein complex would explain all of the observed results.

Ability of Different Fractions of Serum to Activate Coconut Oil—Serum was fractionated by ethanol and in the ultracentrifuge. The ethanol fractionation procedure described by Russ *et al.* (5) was used. By this method four fractions are obtained from normal human serum which contain the following: Fraction A, Cohn's Fractions IV + V + VI; Fraction B, Cohn's Fraction II; Fraction C, Cohn's Fraction III_0; and Fraction D, Cohn's

Fraction I + $III_{1,2,3}$. These fractions were dialyzed for 24 hours at 2° against 0.15 M NaCl and then assayed for their ability to activate coconut oil. The only fractions with any activity were Fractions A and C (Table III, Experiment 2). Fraction A contains the α-lipoproteins, serum albumin, and some glyco- and mucoproteins as minor constituents. Fraction C contains the β-lipoproteins. Since serum albumin is not able to activate coconut oil (see Table I), it would seem most probable that the α-lipoproteins were responsible for the activity of Fraction A.

TABLE III

Ability of Different Serum Fractions to Activate Coconut Oil

Experiment No.	Fraction	Serum equivalent	Glycerol produced
		ml.	*μmole*
1	None	0	0.06
2	A	0.01	0.24
	B	0.01	0.08
	C	0.01	0.17
	D	0.01	0.08
3	α_1-Lipoproteins	0.1	0.30
	β-Lipoproteins	0.5*	0.13
	Residual serum	0.1	0.06
4	Whole serum	0.2	0.86
	α_1-Lipoproteins	0.2	0.54
	"	0.4	0.86
	β-Lipoproteins	0.2	0.14
	"	0.4	0.14

The coconut oil was preincubated for 30 minutes at 37° with the fractions indicated before incubation with lipoprotein lipase. For experimental details, see the text.

* This β-lipoprotein preparation was at least partially denatured.

Ultracentrifugal fractionations were performed on the serum of a patient with idiopathic hyperlipemia and on normal human serum by Dr. R. J. Havel. The identical procedure was followed with both samples. The whole serum was centrifuged at 12° for 43 hours at 40,000 r.p.m. in a Spinco model L ultracentrifuge. The plastic centrifuge tube was sliced so that the upper one-fifth of the tube was separated from the rest and the top material was discarded. The solution in the bottom of the tube was adjusted to a density of 1.019 by the addition of solid NaCl and centrifuged for 45 hours at 40,000 r.p.m. The tube was sliced and the top layer removed and discarded. The bottom solution was then adjusted to a density of 1.063 by the addition of solid NaCl and centrifuged for 24 hours at 40,000 r.p.m. After slicing the tube, the upper solution was collected.

This solution contains the β-lipoprotein component of normal, fasting serum identified as the fraction with an S_f of 3 to 8 (2).

The bottom solution was then brought to a density of 1.21 with solid KBr and the solution centrifuged for 48 hours at 40,000 r.p.m. The α-lipoproteins are in the upper solution obtained after slicing the tube (6). The bottom of the tube contains the original serum with only the lipoproteins removed.

The three samples were then dialyzed for 8 hours at 4° against large volumes of 0.15 M NaCl. Both the α_1- and β-lipoprotein fractions migrated as single, homogeneous bands with the proper mobility in paper electrophoresis.

The results obtained with the ultracentrifugal fractions of the idiopathic lipemic serum are given in Experiment 3, Table III. The residual serum, which contained everything originally present except the lipoproteins, was completely unable to activate coconut oil. The α_1-lipoproteins, on the other hand, were able to convert coconut oil into an active substrate for lipoprotein lipase. The results obtained with β-lipoproteins are difficult to interpret since this preparation was visibly denatured to an unknown extent. Even though an aliquot equivalent to 5 times the amount of α_1-lipoproteins assayed was used, the β-lipoprotein sample was only one-third as active.

In Experiment 4, Table III, the results obtained with the ultracentrifugal fractions of normal human serum are listed. In this experiment, neither the α_1- nor β-lipoproteins were at all denatured. It can be seen that 60 per cent of the activity present in the original serum was recovered in the α_1-lipoprotein fraction. The β-lipoproteins were much less active and could account for, at the most, 16 per cent of the activity of the whole serum. It may be concluded, therefore, that the α_1-lipoproteins are responsible for at least the major portion of the activation of coconut oil when it is preincubated with serum.

DISCUSSION

It is generally accepted that, irrespective of the degree of hydrolysis of ingested fat in the small intestine, the hydrolysis products are recombined in the intestinal mucosa and secreted into the lymphatic system as triglycerides (7). This lipide then becomes associated with a small, but extremely significant, quantity of protein to form chylomicrons (8–13).

The data presented in this paper demonstrate that triglycerides (coconut oil) can interact with α_1-lipoproteins to form a complex which is enzymatically indistinguishable from chylomicrons. This might, in fact, be a close approximation of one-half of a cyclic mechanism for the transport of neutral fat, as schematically represented in Fig. 6. As the result of their inter-

action with α_1-lipoproteins, the triglycerides would then be available as substrate for lipoprotein lipase. This enzyme would catalyze the hydrolysis of the triglyceride moiety to fatty acids and glycerol and thereby regenerate the α_1-lipoprotein.

The soluble, β-lipoproteins of low density, which are also substrates for lipoprotein lipase, might be intermediates in the conversion of chylomicrons to α_1-lipoproteins via β-lipoproteins of higher density. Alternatively, two discrete cycles might exist, one in which α_1-lipoproteins and chylomicrons are interconverted and one involving β-lipoproteins of low and high density. In any case, the fundamental changes involved would be the addition of neutral fat to a lipoprotein and its subsequent removal as fatty acid and glycerol. This concept is in agreement with the observations that the administration of heparin to humans results in a decrease in the concentration in the plasma of chylomicrons and β-lipoproteins of low density with a concomitant increase in the concentration of β-lipoproteins of higher

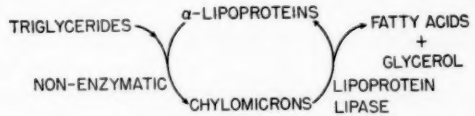


FIG. 6. Hypothetical cyclical mechanism for fat transport

density and α_1-lipoproteins (14–17). There are, of course, differences in the phospholipide and cholesterol content of the various lipoprotein fractions which must be explained by any valid theory, but these changes might occur secondarily as a function of the increase or decrease in neutral fat. At any event, it is clearly indicated that experiments must be performed in which the protein moieties of the several lipoprotein classes are labeled in order to determine which, if any, of the lipoproteins contain the same protein.

SUMMARY

It has been demonstrated that lipoprotein lipase will catalyze the hydrolysis of triglycerides only when they are associated with protein. Coconut oil can interact with lipoproteins to form a lipide-protein complex which is an active substrate for the enzyme. These phenomena have been discussed from the point of view of chylomicron formation and fat transport mechanisms.

The author wishes to thank Dr. Christian B. Anfinsen for many stimulating discussions during the course of this work.

XUM

BIBLIOGRAPHY

1. Korn, E. D., *J. Biol. Chem.*, **215**, 1 (1955).
2. Gofman, J. W., Jones, H. B., Lindgren, F. T., Elliott, H. A., and Strisower, B., *Circulation*, **2**, 161 (1950).
3. Hollett, C., Cole, W. E., and Meng, H. C., *Federation Proc.*, **12**, 70 (1953).
4. Brown, R. K., Boyle, E., and Anfinsen, C. B., *J. Biol. Chem.*, **204**, 423 (1953).
5. Russ, E. M., Eder, H. A., and Barr, D. P., *Am. J. Med.*, **11**, 468 (1951).
6. Lewis, L. A., Green, A. A., and Page, I. H., *Am. J. Physiol.*, **171**, 391 (1952).
7. Frazer, A. C., *Physiol. Rev.*, **20**, 561 (1940).
8. Frazer, A. C., *Discussions Faraday Soc.*, **6**, 81 (1949).
9. Laurell, C. B., *Scand. J. Clin. and Lab. Invest.*, **6**, 22 (1954).
10. Swahn, B., *Scand. J. Clin. and Lab. Invest.*, **5**, suppl. 9 (1953).
11. Elkes, J. J., Frazer, A. C., and Stewart, H. C., *J. Physiol.*, **95**, 68 (1939).
12. Frazer, A. C., and Stewart, H. C., *J. Physiol.*, **95**, 5P (1939).
13. Frazer, A. C., and Stewart, H. C., *J. Physiol.*, **95**, 7P (1939).
14. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., and Simonton, J., *Circulation*, **4**, 465 (1951).
15. Anfinsen, C. B., Boyle, E., and Brown, R. K., *Science*, **115**, 583 (1952).
16. Boyle, E., Bragdon, J. H., and Brown, R. K., *Proc. Soc. Exp. Biol. and Med.*, **81**, 475 (1952).
17. Nikkilä, E., *Scand. J. Clin. and Lab. Invest.*, **5**, suppl. 8 (1953).

FORMIC ACID METABOLISM IN BARLEY LEAVES*

By N. E. TOLBERT

(From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee)

(Received for publication, November 23, 1954)

Formic acid is incorporated by animals into certain positions of characteristic products for this 1-carbon substrate, such as the β-carbon of serine. In plants, an active formic acid dehydrogenase has been reported (1, 2) which should result in a rapid conversion of formic acid to CO_2, followed by photosynthetic CO_2 fixation. Thus feeding of C^{14}-labeled formic acid to plants should result in the appearance of the label in a multiplicity of compounds. Labeling of certain compounds from long time feeding of formic acid to plants has been investigated. Krotkov *et al.* (3) have reported on the labeling in glucose after formic acid is fed to tobacco leaves for 24 hours, Bregoff and Delwiche[1] on the contribution of formate to choline and betaine, and Byerrum *et al.* (4, 5) on the contribution of formate to methionine and lignin.

In the present investigation, formic acid-C^{14} was fed to barley leaves for short periods of time and under varying environmental conditions, and the compounds into which it was incorporated were analyzed by paper chromatography. Formic acid was incorporated directly into the β-carbon of serine and into choline and betaine, as well as into several unidentified compounds. A comparison has been made of the labeling in photosynthetic products from formic acid fixation with the labeling from $C^{14}O_2$ fixation in the light.

Procedure

The C^{14}-labeled potassium formate[2] used in these experiments was prepared by catalytic reduction of $KHC^{14}O_3$ by the method of Melville *et al.* (6). The specific activity of the formate was 8 mc. per mmole. For experiments on CO_2 fixation $NaHC^{14}O_3$ was prepared from $BaC^{14}O_3$ containing 83 μc. per mg.

Wintex barley leaves have been used throughout these experiments, since this barley was used in earlier investigations on the formation of serine from glycolic acid-C^{14} in plants (7). In that work rapid labeling of the

* Work performed under contract No. W-7405-Eng-26 for the Atomic Energy Commission.

[1] Presented before the American Institute of Biological Sciences, Gainesville, Florida, September, 1954.

[2] Kindly furnished by Dr. John R. Totter.

serine occurred in such a manner as to indicate that an active C_1 fragment was derived from glycolic acid. It also developed during the course of the present investigation that with $C^{14}O_2$ as the substrate the large amount of serine present becomes labeled rapidly. Thus the Wintex barley is favorable for the study of $C^{14}O_2$ and $HC^{14}OOK$ incorporation into serine.

The plants were grown in soil in the greenhouse and cut off at the base immediately before use. Leaves were set in a highly radioactive aqueous solution containing the formic acid-C^{14} so that only the bottom of the leaf was in the solution. With a thin window laboratory monitor, considerable C^{14} activity could be detected in the leaf tops within about 2 minutes after they had been placed in the solution. The leaves, with bases dipping into the formate solution, were kept in a glass chamber with a circulating atmosphere which could be varied, and the whole chamber was surrounded by a water cooling bath. When illumination was employed, intensities of 500 foot-candles or higher were obtained from a Photoflood bulb. At the end of the experiment, the leaves were ground in liquid nitrogen, the powder was taken up in water, and the solution immediately heated in a boiling water bath. This killing procedure is sufficiently rapid to prevent appreciable phosphatase action on phosphate esters. After centrifugation to remove the water-insoluble residue, the supernatant fluid was evaporated *in vacuo* to a small volume.

Products formed in the plant from the formate were identified by two-dimensional paper chromatography (8). The solvents were phenol-water in the first direction and butanol-propionic acid-water in the second direction. Products volatilized by this treatment, including formate itself which was lost on drying the acidic paper chromatograms, would not be detected. Compounds were first located by radioautographs made with "no-screen" x-ray film, after which the spots were counted with an end window Geiger tube. The activity of each spot is expressed in Table I as percentage of the total activity on the chromatogram, each vertical line representing one chromatogram. To show the location of the radioactive compounds on the chromatograms, one such radioautograph from $NaHC^{14}O_3$ and one from $HC^{14}OOK$ fixation in the light and in the air are presented in Figs. 1 and 2. The compounds detected by their radioactivity were identified by specific color spray tests (9) and by elution and cochromatography with known compounds. Ninhydrin spray was used to locate amino acids. Organic acids were rechromatographed with carriers in ether-acetic acid-water solvent (10) and located by a bromocresol green spray. The labeled sucrose spot was eluted with water, part of the eluate was rechromatographed with sucrose, and part was hydrolyzed with Dowex 50 in the acid form for 30 minutes at 100°; the mixture was then rechromatographed with carrier glucose and fructose. Glucose and fructose were identified by their R_F

values by the method of chromatography used for photosynthesis (8) and by rechromatography with carrier. The sugars were detected by aniline-trichloroacetic acid spray (11); their radioactivity was found to coincide with that of added carriers. Phosphate esters of organic molecules were not well separated by the chromatographic procedure and are listed only as an area.

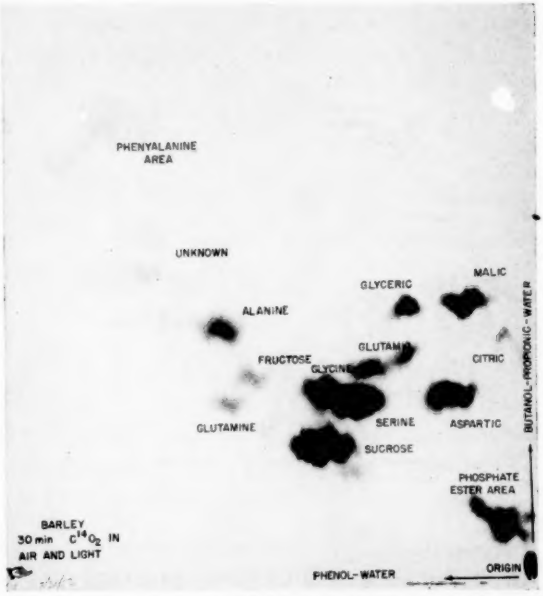


FIG. 1. Chromatogram of $C^{14}O_2$ fixation by Wintex barley leaves

Degradation of the glyceric acid and serine was performed on samples which had been eluted from the paper chromatograms and rechromatographed separately to assure that they were not contaminated. The rechromatographed compounds were combined with carrier and degraded by oxidative decarboxylation with periodic acid (12, 13).

RESULTS AND DISCUSSION

The major products of formic acid fixation over short periods of time under various environmental conditions are reported in Table I. Some of the products formed from formic acid in the light were similar to those from photosynthetic CO_2 fixation, namely, sucrose; phosphate esters of glucose, fructose, and glyceric acid; alanine; serine; and malic, glyceric, and aspartic

FIG. 2. Chromatogram of $HC^{14}OOK$ fixation by Wintex barley leaves

TABLE I

Products from Formic Acid-C^{14} Metabolism by Barley Leaves

All figures as percentage total C^{14} incorporated by the leaf. Small amounts of many other compounds account for the percentage incorporation not listed.

Products	Light and air			Dark and air		Light and nitrogen		
	2 min.	15 min.	40 min.	2 min.	60 min.	5 min.	15 min.	60 min.
Glyceric acid	30	24.9	14.3	0	0.4	23.2	8.4	4.4
Alanine	Trace	1.7	3.9	0	2.4	17.1	11.3	21.9
Serine	40	35.3	7.4	50	28.2	3.7	7.4	3.3
Glycine	10	11.8	1.5	Trace	0	Trace	Trace	Trace
Aspartic acid	5	6.3	24.7	"	10.9	8.5	11.3	3.6
Phosphate esters	5	2.9	6.2	0	0	18.3	19.6	20.3
Sucrose	0	Trace	25.0	0	0	9.8	13.5	21.4
Unknown 1	Trace	"	1.7	Trace	28.2	Trace	4.8	8.1
" 2	10	2.5	Trace	50	2.8	8.5	5.1	3.6

acids. Control experiments were run with $NaHC^{14}O_3$, and fixation into these products showed the same distributional pattern, except that there was a great deal more free glyceric acid from formate than from CO_2 fixation. This can be seen by comparison of the radioautographs in Figs. 1 and 2.

Results from degradation of the free glyceric acid and serine produced in the formate fixation in the light are presented in Table II. The limitation of this type of experiment with higher plants was the length of time necessary for feeding such substrates, which was so great as to result in an approach to uniform distribution into the metabolic pathways of the first C^{14} incorporated. In this respect a nitrogen atmosphere, present only dur-

TABLE II

Specific Activity of Each Carbon Atom of Serine and Glyceric Acid*

Formula	$HC^{14}OOK$, 15 min. in light and air	$HC^{14}OOK$, 15 min. in light and N_2
Serine		
Carboxyl carbon	1.2	3.0
α-Carbon	2.4	4.5
β-Carbon	2.4	6.0
Glyceric acid		
Carboxyl carbon	3.0	1.56
α-Carbon	3.0	0.36
β-Carbon	2.4	0.48

* Expressed as counts per minute per mg. of $BaCO_3$.

ing the feeding time, gave the clearest results. Though the short nitrogen exposure slowed down both $NaHC^{14}O_3$ and $HC^{14}OOK$ fixation, the chromatographic patterns of products did not appear disturbed, since strict anaerobic conditions were not obtained. The distribution of the C^{14} from the formate into serine was highest in the β-carbon, as would be expected from direct incorporation (14), and in the carboxyl carbon of glyceric acid. The latter distribution may represent formate conversion to CO_2 and then photosynthetic fixation into the carboxyl carbon of phosphoglyceric acid. Therefore, if a direct pathway from serine to glyceric acid or triose existed, the hexoses would be expected to have more label in the 1,6-carbons from formate-C^{14} fixation than from $C^{14}O_2$ fixation. This possibility was indicated by Krotkov *et al.* (3), who degraded glucose from tobacco leaves fed formate-C^{14} for 24 hours and found equal distribution of C^{14} in the 3,4 and 1,6 positions, but much less in the 2,5 position.

Our data alone on short time formate fixation do not indicate a direct pathway between glyceric acid and serine. Formate was rapidly fixed in

the dark and in the air into serine (Table I), but only very slowly and in small amounts into glyceric acid, thus indicating no rapid pathway in the dark between these two compounds. However, the pathway from serine to glyceric acid through hydroxypyruvic acid necessitates a reduction (15), and in the dark this may have been very slow in the absence of any photosynthetic reducing power. The formate fixation in the dark for the shortest experiments was entirely by direct incorporation as formate into serine and unknown compounds, and C^{14} did not appear in the carboxylic acids, as would be expected from CO_2. After slightly longer periods of time in the dark, considerable amounts of C^{14} from the formate also appeared in aspartic acid, but not in the organic acids of the Krebs cycle, which suggested C^{14} fixation into aspartic acid also as formate and not as CO_2. In fact, the C^{14} from formate was not incorporated in the dark into the carboxyl group of malic or citric acid for experiments lasting only 1 or 2 hours.

These results suggest that one pathway for formic acid fixation in the light might have been via the CO_2 fixation pathway and phosphoglyceric acid. However, the large amount of free glyceric acid from formate fixation in comparison to CO_2 fixation remains unexplained, unless there was a direct fixation of formate into a "C_2 complex" resulting in a free glyceric acid molecule rather than a phosphorylated one produced during CO_2 fixation. Such a pathway might be more probable with formate because of formate's higher state of reduction compared to CO_2.

Rôle of Photosynthesis in Stimulating Formate Fixation—The fixation of formate into serine in the dark was small when compared to the fixation in light (Table III), although the amount of serine labeled was still very substantial. However, in the dark and in a nitrogen atmosphere, the incorporation of formate into serine was negligible. These data provide additional evidence that the fixation of formate requires energy from oxidative phosphorylation. In the dark and in the air, energy from aerobic metabolism results in a moderate rate of fixation of formate into the β-carbon of serine, but in the light, where the photosynthetic process can make a major contribution to the high energy reservoir, the fixation of formate into the β-carbon of serine progressed very rapidly. This appears to be an example of the utilization by the plant of light energy after its conversion into biochemical energy. It indicates that in the plant other energy-requiring processes besides CO_2 fixation have ready access to the photosynthetically produced energy pool.

Unidentified Compounds Formed from Formic Acid-C^{14}—Many compounds were heavily labeled with C^{14} from feeding barley leaves formic acid-C^{14} for periods of 12 hours or more. Betaine and choline became labeled, but most of the other compounds have not been identified. Two compounds, designated as Unknowns 1 and 2, however, were labeled by formic acid-C^{14}

so rapidly under all the conditions tested (except anaerobically in the dark) that further investigation of them has been made. Unknown 2, having an R_F in water-saturated phenol of 0.8, was labeled as fast as serine in the shortest experiments, but its pool size was very small (Table I). Thus with longer periods of formic acid-C^{14} incorporation, the percentage of the C^{14} in Unknown 2 diminished to a mere trace, but the total amount of C^{14} in it remained constant. The data suggest that Unknown 2 is an active intermediate in the metabolism of formic acid. It does not cochromatograph with any products chosen because of their plausibility and somewhat similar R_F characteristics; *e.g.*, creatinine, creatine, histamine, choline, betaine, sarcosine, or ethanolamine.

Unknown 1 was labeled more slowly than Unknown 2, but over a period of several hours a considerable amount of C^{14} from the formate appeared in it (Table I). Unknowns 1 and 2 were not labeled during a comparable period of $C^{14}O_2$ fixation in the light or dark, but were labeled rapidly with formate, indicating that the formate was being incorporated directly into the unknowns rather than through CO_2. Unknown 1 was also labeled with P^{32} phosphate in the leaf and was hydrolyzed by phosphatase. Upon phosphatase hydrolysis, all of the C^{14} appeared in a new compound with an R_F value of 0.95 in phenol and 0.7 in butanol-propionic acid-water. As with Unknown 2, Unknown 1 and its hydrolysis products did not cochromatograph with any compounds tested, especially those involved in phospholipide synthesis.

TABLE III

Relative Amounts of Formate Fixation into Serine

Experimental conditions	Total formate-C^{14} fixed	Total serine-C^{14}
	c.p.s.	*c.p.s.*
Light-air	23.8	8.4
Dark-air	4.8	1.3
Dark-N_2	1	Trace

A Wintex barley leaf fixed formate-C^{14} for a 15 minute period for each environmental condition, and an equal aliquot of plant tissue from each was chromatographed. The values were taken directly from each chromatogram and are uncorrected for absorption of radioactivity by the paper.

SUMMARY

Two major sites of formate-C^{14} fixation in Wintex barley leaves in the light were into the carboxyl group of glyceric acid and into the β-carbon of serine. Both pathways utilized light energy, while fixation into the β-car-

bon of serine also proceeded in the dark, though at a slower rate. Formate was incorporated into two unidentified compounds as rapidly as into serine.

The author acknowledges the capable assistance of Mrs. Betty Ann Gwin throughout most of this work.

BIBLIOGRAPHY

1. Mathews, M. B., and Vennesland, B., *J. Biol. Chem.*, **186,** 667 (1950).
2. Davison, D. C., *Biochem. J.*, **49,** 520 (1951).
3. Krotkov, G., Vittorio, P. V., and Reed, G. B., *Arch. Biochem. and Biophys.*, **51,** 147 (1954).
4. Byerrum, R. U., Flokstra, J. H., Dewey, L. J., and Ball, C. D., *J. Biol. Chem.*, **210,** 633 (1954).
5. Byerrum, R. U., Hamill, R. L., and Ball, C. D., *J. Biol. Chem.*, **210,** 645 (1954).
6. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169,** 419 (1947).
7. Tolbert, N. E., and Cohan, M. S., *J. Biol. Chem.*, **204,** 649 (1953).
8. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W., *J. Am. Chem. Soc.*, **72,** 1710 (1950).
9. Block, R. J., LeStrange, R., and Zweig, G., Paper chromatography, New York (1952).
10. Denison, F. W., and Phares, E. F., *Anal. Chem.*, **24,** 1628 (1952).
11. Hough, L., Jones, J. K. N., and Wadman, W. H., *J. Chem. Soc.*, 1702 (1950).
12. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139,** 687 (1941).
13. Bassham, J. A., Benson, A. A., and Calvin, M., *J. Biol. Chem.*, **185,** 781 (1950).
14. Welch, A. D., and Nichol, C. A., *Ann. Rev. Biochem.*, **21,** 633 (1952).
15. Stafford, H. A., Magaldi, A., and Vennesland, B., *J. Biol. Chem.*, **207,** 621 (1954).

XUM

THE FORMATION OF SERINE FROM PYRUVATE*

By JOSEPH F. NYC and IRVING ZABIN

(From the Department of Physiological Chemistry, University of California School of Medicine, Los Angeles, California)

(Received for publication, December 4, 1954)

It has been recognized for many years that the amino acid serine can be formed from carbohydrate. More recently, tracer studies have shown that acetate and pyruvate can be converted to serine (1, 2), but the details of this mechanism are not clear. It has been suggested (2), however, that pyruvate is converted to serine by a reversal of the deamination reaction of serine which occurs in *Escherichia coli* (3).

We have studied the conversion of carboxyl- and methyl-labeled acetate and of β-labeled pyruvate to serine in the rat. In order to obtain a more complete picture of events, two additional amino acids, alanine and glycine, were also isolated and examined. The results obtained show that pyruvate is not directly transformed to serine, but that the 3-carbon chain of pyruvate is altered prior to the formation of the amino acid.

EXPERIMENTAL

Administration of Isotope—20 mmoles of acetate-1-C^{14}, specific activity 4×10^6 c.p.m. per mg. of C, were injected intraperitoneally into twenty weanling rats in daily doses over a 4 day period. 14 mmoles of acetate-2-C^{14}, specific activity 3.35×10^6 c.p.m. per mg. of C, were similarly injected into twenty rats. Each group of animals was sacrificed 24 hours after the last injection.

Pyruvic acid-3-C^{14} was prepared by hydrolysis of pyruvamide-3-C^{14} (4) (Nuclear Instrument and Chemical Corporation). Assay after hydrolysis (5) indicated an almost quantitative conversion to the keto acid. The neutralized acid, 1.1 mmoles, specific activity 4×10^6 c.p.m. per mg. of C, was injected into six animals in two doses at a 24 hour interval. This group was sacrificed on the 3rd day. Pyruvate was administered to two additional groups of two young rats each. One group was given 0.35 mmole, and the animals were sacrificed 3 hours later. The second group was given 0.1 mmole of pyruvate and was sacrificed at the end of 1 hour.

Isolation and Degradation of Amino Acids—Visceral organs and the brain were removed from the group of animals given acetate-1-C^{14}, and

* This investigation was supported by research grants from the International Minerals and Chemical Corporation and from the National Institutes of Health, Public Health Service.

amino acids were isolated from the carcass. In all other experiments the amino acids were isolated from the whole animal, except for the brain which was removed for other purposes.

After thorough extraction of the ground animal tissues with ether and ethanol, the residual material was heated under reflux with 6 N HCl for 18 hours. Aromatic and acidic amino acids were removed from the hydrolysate by absorption on activated charcoal and Amberlite IR-4B, respectively. Alanine, glycine, and serine were separated on a column of Dowex 50 ion exchange resin with 1.5 N HCl according to Stein and Moore (6). Alanine was converted to the azobenzene sulfonate, serine to the *p*-hydroxyazobenzene-*p'*-sulfonate, and glycine to the 5-nitronaphthalene-1-sulfonate (7). Each compound was recrystallized from water to a constant specific activity. Treatment with barium ion released the free amino acids, which were further checked for purity by paper chromatography and isotope assay.

Alanine was degraded as described by Gilvarg and Bloch (8), serine was treated by the method of Sakami (9), and glycine was decomposed according to Weissbach and Sprinson (10) in order to obtain specific activities of individual carbon atoms. The specific activity of the β-carbon of alanine was calculated by difference; all others were determined directly.

Isotope Analyses—Formaldehyde dimethone was plated directly. Other samples were converted to $BaCO_3$. In a few cases the dimethone derivatives were also converted to $BaCO_3$. Assays and corrections were carried out by standard methods.

Results

Acetate-1-C^{14}—The administration of carboxyl-labeled acetate to a group of animals resulted in the formation of alanine, serine, and glycine which contained isotopic carbon only in the carboxyl positions (Table I). The specific activity of C^{14} in carbon atom 1 of alanine was found to be higher than that in the corresponding carbon atom of serine, and this in turn was higher than in the carboxyl carbon of glycine. These data are in complete agreement with earlier work which suggested a biosynthetic pathway requiring pyruvate as an intermediate in the conversion of acetate to glycine via serine (2).

Acetate-2-C^{14}—The distribution of isotope in the three amino acids isolated after injection of methyl-labeled acetate is shown in Table I. It may be noted that the specific activities of carbon atoms 2 and 3 of alanine are almost identical, and that this is also true for carbons 2 and 3 of serine. The carboxyl carbon atoms contain a level of activity of about one-half that of carbon atoms 2 or 3 in each case. This pattern is to be expected, since pyruvate formed from acetate-2-C^{14} through the Krebs cycle will be

labeled in all 3 carbon atoms, with equal activity at carbons 2 and 3 and less activity at carbon 1 (11).

It was also found, however, that the specific activity in each carbon atom of serine was about 50 per cent higher than in each of the corresponding carbon atoms of alanine. This is in contrast to the results obtained with

TABLE I

Distribution of Isotope in Amino Acids from Acetate-1-C^{14} and Acetate-2-C^{14}

Amino acid	Activity, c.p.m. per mg. C					
	Acetate-1-C^{14}			Acetate-2-C^{14}		
	Carbon atom No.					
	1	2	3	1	2	3
Alanine	1255	30	10	405	925	920
Serine	795	35	35	615	1290	1340
Glycine	665	10		490	935	

TABLE II

Distribution of Isotope in Amino Acids from Pyruvate-3-C^{14}

Amino acid	Activity, c.p.m. per mg. C								
	48 hrs.			3 hrs.			1 hr.		
	Carbon atom No.								
	1	2	3	1	2	3	1	2	3
Alanine	1100	2400	11,860	695	1680	10,460			
Serine	440	1230	1,335	280	1075	1,280	75	230	220
Glycine	315	655		135	290				

acetate-1-C^{14} and suggests that the methyl carbon of acetate is a more direct precursor of serine than is pyruvate itself.

Pyruvate-3-C^{14}—Three experiments, which differed in the time of sacrifice after administration of labeled pyruvate, were performed. The first experiment was over a 48 hour period, the second over a 3 hour period, and the third experiment over a 1 hour period. Only serine was isolated in the third experiment.

The distribution of tracer is shown in Table II. Alanine was marked primarily in the β-carbon atom, but serine was, within experimental error, equally labeled at carbon atoms 2 and 3. Furthermore, each of these

labeled atoms had a specific activity of about one-tenth that of the β-carbon atom of alanine. While the isotope distribution in alanine reflects that in administered pyruvate, the pattern in serine is similar to that obtained after injection of methyl-labeled acetate.

DISCUSSION

Previously available evidence has supported the hypothesis that pyruvate is a direct precursor of serine. This evidence has been based on the work of Shemin (12), who has shown that serine is converted to glycine by removal of carbon atom 3, and on the isotope distribution found in glycine after the administration of acetate or pyruvate. Anker administered pyruvate labeled at the carbonyl position to rats and isolated glycine labeled primarily at the α-carbon atom (1). Arnstein and Neuberger gave acetate-2-C^{14} and obtained glycine labeled in the same way (2). From these data, it had reasonably been concluded that acetate is converted to pyruvate and pyruvate directly transformed to serine. It may be noted here that Anker's experiment rules out a conversion of pyruvate to acetate prior to the formation of glycine, because pyruvate-2-C^{14} would become acetate-1-C^{14}, and this latter compound could only form glycine labeled at the carboxyl position, a fact that has been shown to be true previously and here also (Table I).

The pattern of tracer found in serine, alanine, and glycine from acetate-2-C^{14} and pyruvate-3-C^{14} in the experiments reported here, however, strongly supports the conclusion that the 3-carbon chain of pyruvate is not a direct precursor of serine. The major point of evidence is simply that pyruvate-3-C^{14} gives rise to serine equally labeled at carbon atoms 2 and 3. Alanine, on the other hand, was found to have most activity at carbon 3. The 3-carbon chain of pyruvate must therefore have been altered prior to the formation of serine. A second significant finding concerns the relative levels of activity in serine and alanine after administration of acetate-2-C^{14}. In this case carbon 3 of alanine was only about two-thirds as active as carbon atom 2 or 3 of serine. Alanine may be expected to be an index of the isotope concentration in pyruvate during the experiment. If pyruvate were a direct source of serine, the isotope concentration in serine derived from acetate-2-C^{14} should approach the level in alanine. The reverse situation was actually observed, indicating that acetate is a more effective precursor of serine than is pyruvate.

It can be argued that serine is in fact formed directly from pyruvate, but that during the experimental period an initial high level of activity at carbon atom 3 of serine is lowered by breakdown and formation of new serine. This alternative interpretation of the data would seem very unlikely, however, because even after an experimental period of only 1 hour

pyruvate-3-C^{14} administration resulted in serine symmetrically labeled at carbons 2 and 3. The reaction demonstrated by Sakami, the formation of serine from glycine and formate (13), likewise cannot explain these results. In every case, the isotope concentration of glycine was lower than that in serine. It is apparent that under these conditions serine does not arise from glycine, but is instead a precursor of glycine. A similar conclusion has previously been reached by Arnstein and Neuberger (14).

Recently, Meister *et al.* demonstrated the enzymatic conversion of hydroxypyruvate to serine (15), and Stafford *et al.* found an enzyme in plant tissues which reversibly converts hydroxypyruvate to glyceric acid in the presence of reduced diphosphopyridine nucleotide (16). These results suggest that pyruvate might be transformed to hydroxypyruvate and serine through glycolytic intermediates. This pathway, however, must also be ruled out, because a reversal of glycolysis could not form serine equally labeled at carbon atoms 2 and 3 from pyruvate labeled only at carbon 3. The data presented here are consistent with the following suggestion: pyruvate is carboxylated to a 4-carbon compound which is further converted through one or more symmetrical intermediates to hydroxypyruvate and finally to serine.

SUMMARY

Acetate-1-C^{14}, acetate-2-C^{14}, and pyruvate-3-C^{14} were administered to weanling rats, and alanine, serine, and glycine were isolated and degraded.

Acetate-2-C^{14} contributed more isotope to serine than to alanine. Pyruvate-3-C^{14} gave alanine labeled primarily in the β-carbon atom, but serine with equal activities at carbon atoms 2 and 3. These results show that the carbon chain of pyruvate is not directly converted to serine. The existence of a pathway involving a symmetrical intermediate is suggested.

The authors wish to express their appreciation to Miss Nancy Wilcox and Miss Aniko Gaal for expert technical assistance.

BIBLIOGRAPHY

1. Anker, H. S., *J. Biol. Chem.*, **176,** 1337 (1948).
2. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **45,** p. iii (1949).
3. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **148,** 249 (1943).
4. Anker, H. S., *J. Biol. Chem.*, **176,** 1333 (1948).
5. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943).
6. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 179 (1949).
7. Stein, W. H., Moore, S., Stamm, G., Chou, C.-Y., and Bergmann, M., *J. Biol. Chem.*, **143,** 121 (1942).
8. Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **193,** 339 (1951).
9. Sakami, W., *J. Biol. Chem.*, **187,** 369 (1950).

XUM

10. Weissbach, A., and Sprinson, D. B., *J. Biol. Chem.*, **203,** 1023 (1953).
11. Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, **176,** 1263 (1948).
12. Shemin, D., *J. Biol. Chem.*, **162,** 297 (1946).
13. Sakami, W., *J. Biol. Chem.*, **176,** 995 (1948).
14. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **55,** 271 (1953).
15. Meister, A., Fraser, P. E., and Tice, S. V., *J. Biol. Chem.*, **206,** 561 (1954).
16. Stafford, H. A., Magaldi, A., and Vennesland, B., *J. Biol. Chem.*, **207,** 621 (1954).

METABOLISM OF METHIONINE-$C^{14}H_3$ IN THE CHICK

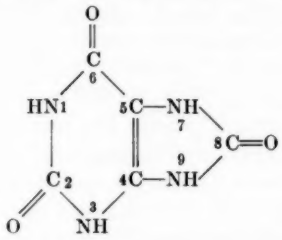
II. DISTRIBUTION OF C^{14} IN THE URINARY URIC ACID MOLECULE*

By JOHN T. SIME† and B. CONNOR JOHNSON

(From the Division of Animal Nutrition, Department of Animal Science, University of Illinois, Urbana, Illinois)

(Received for publication, June 28, 1954)

In the biosynthesis of uric acid by the bird, it has been shown that carbon 6 results from the fixation of CO_2 (1), that glycine is incorporated *in toto* into the molecule as carbons 4 and 5 and nitrogen 7 (2–5), and that carbons 2 and 8 can be derived from formate (6–9).



In studying the over-all metabolism of methyl-C^{14}-methionine in the chick, it has been found that the uric acid excreted was radiocarbon-labeled, and that the distribution of the label in the molecule conforms with the concept of the methionine methyl being a precursor of the "active 1-carbon compound."

EXPERIMENTAL

Three chicks (Columbian female, New Hampshire male, cross) were raised to 5 weeks of age on a diet[1] adequate in all known nutrients. They were housed in a battery cage on raised wire floors and given food and water

* Supported in part by a grant-in-aid from the United States Atomic Energy Commission under contract No. AT(11-1)-67, Animal Science project 2.

† Present address, University of Arkansas School of Medicine, Little Rock, Arkansas.

[1] The composition of this diet in per cent was corn 55.78, soy bean oil meal (50 per cent protein) 34, bone meal 3.0, limestone 1.0, salt 0.5, vitamin A and vitamin D oil 0.05, $MnSO_4$ 0.05, alfalfa 1.0, DL-methionine 0.10, Aurofac 1.0, riboflavin concentrate (1500 γ per gm.) 0.02, corn distillers' solubles 4.0. This ration was supplemented with niacin (1 gm. per 100 pounds) and choline (24 gm. per 100 pounds).

ad libitum. At 5 weeks, operations were performed on each bird so that it was possible to collect urine uncontaminated with fecal material.

The operation is a modification of the method devised by Hester *et al.* (10), and is simple and seems to incapacitate the bird very little. The cloaca is cut laterally and the upper flap fastened either by surgical staples or suture to the pygostyle. To this is attached a piece of Tygon tubing ($\frac{1}{2}$ inch) by means of adhesive tape. This directs the flow of the urine into a rubber prophylactic which is attached to the tube by means of a rubber band.

This method is suitable for 36 hours of collection, the collection bag being emptied every 12 hours. After 36 hours, leakage may occur due to tissue shrinkage in the healing process. However, this was sufficient time for our experiment.

The chicks were injected intraperitoneally (8 hours postoperative) with 42 mg. of methionine-$C^{14}H_3$ (specific activity 244 μc. per mmole). They were placed in individual raised wire bottom cages, and 36 hour urine collections made. Filtering the urine removed the uric acid, which was purified by recrystallization (11). The uric acid was degraded by a slight modification of earlier procedures (12–14) in order to determine the relative radioactivity of the individual carbon atoms in the molecule. In the degradation procedure of Sonne, Buchanan, and Delluva (13, 14), carbon 6 is removed as CO_2, carbons 2 and 8 as urea, and carbons 4 and 5 as glyoxylic acid in the form of the semicarbazone. Due to the great instability of glyoxylic acid, yields are poor for this step. Since we did not care to distinguish between carbons 4 and 5, we modified the procedure to allow the glyoxylic acid to oxidize completely to oxalic acid. This modification eliminated the lead precipitation and semicarbazide steps, the oxalic acid being precipitated and isolated as the calcium salt. In order to determine the respective activities of carbons 2 and 8, the method of Nightingale (12) was used. The uric acid molecule was degraded to alloxantine by potassium chlorate and HCl oxidation resulting in the removal of carbon 8 as urea. The difference in specific activity between alloxantine and uric acid gave then the activity of carbon 8. The carbon 2 activity was readily determined by the difference between the activity of carbon 8 and the combined activities of carbons 2 and 8.

The radioactivity of each carbon fragment was determined by converting the sample to CO_2 by wet combustion and measuring the CO_2 activity in the vibrating Reed electrometer.

RESULTS AND DISCUSSION

The radioactivity of the carbon atoms of uric acid is given in Table I. As can be seen, carbons 2 and 8 contain 92.4 per cent of the original activ-

ity in the uric acid. The urinary uric acid contains 9.3 per cent of the activity originally administered. These figures are similar to those obtained by Elwyn and Sprinson (15) and Marsh (8), using formate as a precursor. Elwyn and Sprinson found, using formate in pigeons, 96.5 per cent of the total uric acid activity in carbons 2 and 8, 3.2 per cent in carbons 4 and 5, and 0.46 per cent in carbon 6. Marsh, also using formate in pigeons, found that carbons 2 and 8 contained 89 per cent as compared to 8.7 per cent for carbons 4 and 5 and 2.25 per cent for carbon 6. According to the work of Reid and Landefeld (16), 91 per cent of the radioactivity in the excreted uric acid was located in the ureido carbons after administration of histidine-2-C^{14}. The results of the present study with methionine as the precursor of uric acid in the chick demonstrate the reversibility of methyl synthesis from the "active 1-carbon compound."

TABLE I

*Degradation of Uric Acid Isolated from Chick Urine during 36 Hours following One Injection of Methionine-$C^{14}H_3$**

Position	Radioactivity found in individual positions of uric acid molecule		
	Uric acid	Activity of total uric acid molecule†	Methyl-C^{14}-methionine* injected
	μc. per mole	*per cent*	*per cent*
Carbon 6	2.5	0.9	0.09
Carbons 4 and 5	18.0	6.7	0.6
" 2 " 8	248.6	92.4	8.7
Carbon 8	98.2	36.5	3.4
" 2	150.4	55.9	5.2

* 244 μc. per mmole (injected methionine).

† 269 μc. per mole (isolated uric acid).

When the ratio of the labeling of carbons 2 and 8 of uric acid from methionine methyl in this work is compared with the ratio obtained from formate, a considerably greater proportion of the label is found in position 2 than in position 8. The ratio of carbon 2 to carbon 8 was 1.53:1, while Elwyn and Sprinson (15) found a ratio of 1 when the β-carbon of serine was the precursor of carbons 2 and 8, as did Marsh (8) and Sonne *et al.* (1), using labeled formate. The 6.7 per cent labeling in position 4 + 5 indicates glycine synthesis from "active 1-carbon fragment" from the methionine methyl.

The authors would like to express their gratitude to the late Dr. K. A. Burke for preparing the methionine-$C^{14}H_3$ and to Dr. H. M. Scott for the chicks used in this experiment.

SUMMARY

It has been shown that the methyl group of methionine serves as a source of "active 1-carbon compound" for the biosynthesis of uric acid by the chick *in vivo*. The C^{14} of the methyl group was incorporated into carbons 2 and 8 of uric acid to the extent of 56 per cent of the uric acid activity in position 2, and 36 per cent in position 8.

BIBLIOGRAPHY

1. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **166,** 395 (1946).
2. Shemin, D., *J. Biol. Chem.*, **162,** 297 (1946).
3. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **167,** 875 (1947).
4. Karlsson, J. L., and Barker, H. A., *J. Biol. Chem.*, **177,** 597 (1949).
5. Goldthwait, D. A., and Peabody, R. A., *Federation Proc.*, **13,** 218 (1954).
6. Greenberg, G. R., *Federation Proc.*, **13,** 221 (1954).
7. Buchanan, J. M., and Sonne, J. C., *J. Biol. Chem.*, **166,** 781 (1946).
8. Marsh, W. H., *J. Biol. Chem.*, **190,** 633 (1951).
9. Buchanan, J. M., and Schulman, M. P., *J. Biol. Chem.*, **202,** 241 (1953).
10. Hester, H. R., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, **128,** 592 (1939–40).
11. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, 729, Philadelphia, 12th edition (1947).
12. Nightingale, D., *Org. Syntheses*, **23,** 6 (1943).
13. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173,** 69 (1948).
14. Buchanan, J. M., Sonne, J. C., and Delluva, A. M., *J. Biol. Chem.*, **173,** 81 (1948).
15. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **184,** 465 (1950).
16. Reid, J. C., and Landefeld, M. O., *Arch. Biochem. and Biophys.*, **34,** 219 (1951).

PROTEOLYTIC ENZYMES OF LUNG*

By ARTHUR M. DANNENBERG, JR.,† AND EMIL L. SMITH

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

(Received for publication, November 8, 1954)

The lung usually is considered to be a passive tissue whose main function is to permit gaseous diffusion to and from the blood; however, the lung is also an organ of defense. Its phagocytes remove inhaled dust particles and bacteria. A considerable part of the lung parenchyma consists of mononuclear phagocytes and potential mononuclear phagocytes. Since these cells are not readily available in quantity, a survey of the proteolytic enzymes of lung was made with the hope that such information could be correlated eventually with the enzymes of the mononuclear phagocytes as well as be helpful in understanding the liquefaction[1] process of tuberculosis.

Fruton (1) demonstrated the presence of leucine aminopeptidase, tripeptidase, and glycylglycine dipeptidase in extracts of rabbit lung. He could detect no hydrolysis of the synthetic substrates for several proteinases, including benzoyl-L-argininamide. Weiss and Halliday (2), however, have reported the presence of an enzyme in rabbit lung which hydrolyzes benzoyl-L-argininamide. Nye (3) and Weiss (4) have also identified a proteinase in lung that is optimally active at pH 3 to 4.

The present study confirms the work of Fruton (1) on the peptidases of lung and reports the presence of two additional peptidases. We have also found that lung extracts show two regions of proteinase activity, one at acid pH (Proteinase I) and another at alkaline pH (Proteinase II). A method of purification and some of the properties of Proteinase I are described. Further studies of its specificity are given elsewhere (5). A synthetic substrate, *N*-acetyl-L-tyrosine ethyl ester, has been found for a component of Proteinase II, but purification of this fraction has not been attempted as yet.

* This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service.

† Postdoctoral Fellow of the National Tuberculosis Association, administered by the National Research Council.

[1] When a tuberculous focus liquefies or softens, the material that it contains is able to spread throughout the lung. If it did not liquefy, the disease would remain localized and would heal in most human cases.

Lung Peptidases

A saline extract of whole fresh lung from Sprague-Dawley white rats or from freshly slaughtered beef was prepared at 5° by grinding the lung with 10 volumes of chilled 0.9 per cent NaCl, bringing the suspension to pH 8 with 0.1 M NaOH, and removing the insoluble residue by centrifugation. Activity towards peptide and amide substrates was estimated by a titration method (6). Extracts were incubated at 39° with buffer, the appropriate activators, and 0.05 M substrate. At intervals, 0.2 ml. aliquots were titrated to the thymolphthalein end-point with 0.01 M KOH in 90 per cent ethanol. Activity is expressed as the first order constant K_1, calculated in decimal logarithms. C_1, the proteolytic coefficient, is defined as K_1/E, where E is the protein N in mg. per ml. of test solution.

TABLE I

Peptidase Activity of Saline Extracts of Rat and Beef Lung

The assays were made with 0.05 M substrate at 39° in 0.02 M tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 7.8 to 8.6. The enzyme concentration was 0.12 mg. of protein N per ml. for the rat and 0.30 mg. of protein N per ml. for beef.

Substrate	C_1 (rat lung)		C_1 (beef lung)	
	Without activator	With 0.002 M Mn^{++}	Without activator	With 0.002 M Mn^{++}
L-Leucinamide	0.013	0.014	0.007	0.033
Glycylglycine	0.045	0.141*	0.012	0.079*
L-Prolylglycine	0.012	0.056	0.008	0.003
Glycyl-L-proline	0.003	0.009	0.001	0.003

* Contained 0.002 M Co^{++} instead of Mn^{++}.

Table I shows the activity of lung extracts towards four compounds which have been used to estimate certain peptidases (7): leucinamide for leucine aminopeptidase, glycylglycine for a dipeptidase, glycylproline for prolidase, and prolylglycine for iminodipeptidase. In general, the known metal activators for these enzymes produce the expected effects. In addition to the activities in Table I, both rat and beef lung possess a highly active tripeptidase, as judged by the rapid hydrolysis of glycylglycylglycine.

Lung Proteinases

Method of Assay—Saline extracts of rat and beef lung were assayed at 39° for proteinases essentially by the method of Kunitz (8), with twice crystallized equine hemoglobin as substrate. The test solution contained 1 ml. of a 2 per cent solution of urea-denatured hemoglobin which con-

tained buffer and 1 ml. of the tissue extract. Proteolysis was stopped by adding 2 ml. of a 5 per cent solution of trichloroacetic acid. Blank solutions were prepared by adding trichloroacetic acid to similar preparations at zero time. Both blank and test solutions were kept at 39° for 1 hour after the addition of trichloroacetic acid and then filtered. 1 ml. aliquots of each filtrate were diluted to 3 ml. with water and their absorption at 280 mμ was determined with a Beckman spectrophotometer. The proteolytic activity is expressed as the optical density increment of the incubated solution minus that of the corresponding blank. It should be noted that it is also necessary to incubate a blank of hemoglobin, activator, and buffer

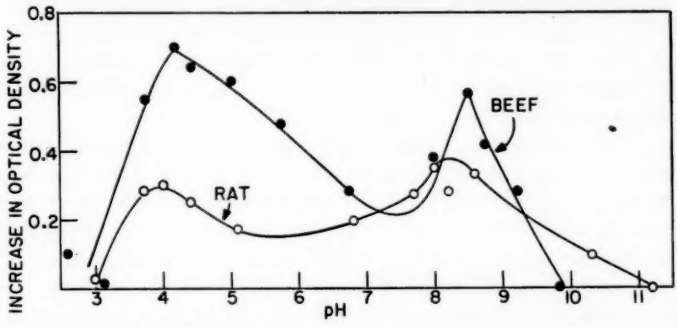


FIG. 1. Hydrolysis of hemoglobin by extracts of fresh rat lung (○) and frozen beef lung (●) as a function of pH. The concentration was 0.63 mg. of protein N per ml. The buffers were 0.1 M citrate below pH 7.0 and 0.1 M Tris (tris(hydroxymethyl)aminomethane) at pH 8.0 and above; a mixture of the two was used between pH 7.0 and 8.0.

without enzyme, when $Fe(NH_4)_2(SO_4)_2$ is used as an activator, because of the possible oxidation of Fe^{++} to Fe^{+++} which absorbs at 280 mμ.

Characterization—In Fig. 1 is given the effect of pH on the hydrolysis of hemoglobin by crude extracts of rat and beef lung. The ordinate shows the increase in optical density at 280 mμ for an incubation of 1 hour. The two peaks suggest the presence of at least two proteinases, Proteinase I with an optimum near pH 4.0 and Proteinase II with an optimum near pH 8.4. Beef lung extract is twice as active as an extract of rat lung.

Purification and Properties of Proteinase I

Assays were made at pH 4.0 with 1 per cent urea-denatured hemoglobin as the substrate, 0.002 M $Fe(NH_4)_2(SO_4)_2$ as the activator, and an incubation period of 15 minutes at 39°. Under these conditions the increase in optical density was linear with enzyme concentration in the range used. The unit of activity is the amount of enzyme which gives an increase in

optical density at 280 mμ of 0.10 in 15 minutes. Purity is estimated by the number of units per mg. of protein N per ml. of test solution.

Step 1: Preparation of Acetone-Dried Powder—8 kilos of fresh beef lung were put through a mechanical meat grinder twice. This material was treated in portions with 2 volumes of acetone at −30° in a Waring blendor for 1 minute and then centrifuged. The extracts were pooled and filtered through gauze. 2 more volumes of cold acetone were added to the crude filtrate and the mixture was allowed to stand overnight at 4°. The clear supernatant fluid was removed and discarded. The sediment was resuspended in 3 to 4 volumes of cold acetone. After 2 hours the wash fluid was discarded, and 1 to 2 volumes of cold acetone were added. The precipitate was collected on a large Büchner funnel, washed several times with cold acetone, and finally with ether. The residue was rapidly air-dried at room temperature and *in vacuo* over P_2O_5. The powder showed no loss of activity when kept for 1 month at 5°.

Steps 2 and 3: Treatment with Acid Followed by Ammonium Sulfate Fractionation—A 2 per cent aqueous extract of the acetone-dried powder was made (pH = 6.8), and the insoluble fraction was removed by centrifugation. A crystal of thymol was added to the solution and also $Fe(NH_4)_2(SO_4)_2$ to a final concentration of 0.004 M. With mechanical stirring at 4°, 0.1 N HCl was added dropwise to pH 3.1, and 1.0 M citrate buffer at pH 3.0 was added to make a final citrate concentration of 0.04 M. The mixture was allowed to remain at 4° for 3 to 7 days. (After 5 weeks there was no further gain in purity, although a slight decrease in yield occurred.)

The precipitate was removed by centrifugation and discarded. Solid $(NH_4)_2SO_4$ was added to 0.45 saturation and the precipitate discarded. More solid $(NH_4)_2SO_4$ was added to 0.8 saturation and the precipitate collected. The precipitate was suspended in a minimal volume of water and dialyzed against water at 4°. A crystal of thymol was added. Such preparations could be stored in a deep freeze with little or no detectable change in activity for several months.

These steps of purification, as well as procedures described below, are summarized for a representative preparation in Table II. Other preparations gave a purification ranging from 110 to 200 times and a yield of 43 to 73 per cent.

Efforts have been made to obtain greater purification of this proteinase. The highest purification achieved was 350 times; this was accomplished by adsorption of the purified Proteinase I (157 times) on calcium phosphate gel in acetate buffer at pH 4.0 at 39°. It was eluted with M/15 phosphate buffer at pH 7.4 at 4°, and the enzyme precipitated at 0.5 saturated $(NH_4)_2SO_4$. After dialysis, the yield was only 2 per cent of the original activity.

Properties of Proteinase I—The enzyme shows good stability in both the

crude and the purified state. The purified enzyme can be kept at 39° at pH 4.6 for 24 hours with only a 16 per cent loss of activity. Activity is a linear function of enzyme concentration over a reasonable range (Fig. 2). Fig. 3 gives the optimum as near pH 3.8, and the absence of activity above

TABLE II

Purity and Yield of Proteinase I from Beef Lung

Assays were performed with hemoglobin as substrate in 0.1 M citrate buffer at pH 4.0. The results are for a representative batch of 8 kilos of fresh bovine lung.

Step	Activity	Total units $\times 10^{-5}$	Yield
	units per mg. protein N per ml.		*per cent*
Aqueous extract	0.175	408	100
Step 1	5.0	520	127
Steps 2 and 3	27.5	296	73
Adsorption on calcium phosphate gel and elution	50.0	16	4
Same, and pptn. with 0.5 saturated ammonium sulfate	61.0	8	2

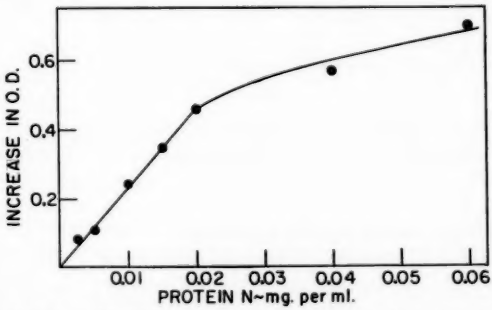


FIG. 2. Activity of purified Proteinase I as a function of enzyme concentration at pH 4.0 with 1 per cent urea-denatured hemoglobin in 0.1 M citrate buffer. The ordinate shows the increase in optical density at 280 mμ.

pH 7.0 indicates that Proteinase II has been removed. As judged by the action on hemoglobin, the preparation purified 150 times is about one-tenth as active as crystalline trypsin.

The activity of crude Proteinase I is enhanced slightly by Fe^{++}, but the purified enzyme is not influenced by this ion. The crude extract is not affected by phosphate, cysteine, Mn^{++}, or Zn^{++}. Ethylenediaminetetraacetate (Versene) at 0.01 M and diisopropyl fluorophosphate (DFP) at 1.4×10^{-4} M do not influence the activity of purified Proteinase I.

Cysteine (0.005 M) increases the activity of the purified enzyme about 20 per cent. Iodoacetamide (0.001 M) inhibits about 50 per cent and *p*-chloromercuribenzoate (0.0001 M) about 40 per cent. These substances were mixed with the enzyme at room temperature at pH 5 to 6 for 30 to 60 minutes before assay. As indicated elsewhere (5), iodoacetamide and *p*-chloromercuribenzoate inhibit completely the ability of Proteinase I at pH 7 to form polymers from amino acid esters, and cysteine activates this reaction. These observations suggest that a sulfhydryl group is essential for both hydrolytic and polymerase activities of this enzyme.

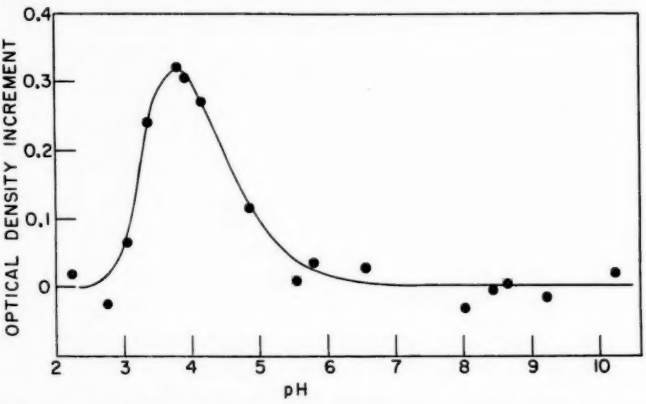


FIG. 3. Activity of purified Proteinase I as a function of pH with 1 per cent urea-denatured hemoglobin as substrate in 0.1 M citrate below pH 7.5 and in 0.1 M Tris at higher pH values.

A number of substances at 0.05 M were tested at pH 4.0 in 0.02 M citrate or acetate buffer as substrates for purified Proteinase I (0.07 mg. of protein N per ml.). The following were used: *N*-acetyl-L-phenylalanyl-L-phenylalanine (pepsin substrate), *N*-acetyl-L-phenylalanyl-L-phenylalanine ethyl ester, L-phenylalanine methyl ester, Cbz-L-tryptophylglycinamide[2] (chymotrypsin substrate), Cbz-glycyl-L-tryptophanamide (chymotrypsin substrate), L-leucinamide, L-leucine ethyl ester, Cbz-L-leucinamide, Cbz-L-leucyl-L-alaninamide, Cbz-L-alanyl-L-leucinamide, Cbz-glycylglycinamide, Cbz-L-glutamic acid diamide (papain substrate), Cbz-glycyl-L-prolylglycine, Cbz-glycyl-L-histidinamide, benzoyl-L-argininamide (trypsin and papain substrate), *N*-acetyl-L-tyrosinamide (chymotrypsin substrate), Cbz-L-glutamyl-L-tyrosine (pepsin substrate), Cbz-L-isoglutamine (papain substrate), and Cbz-glycyl-L-phenylalanine (carboxypeptidase substrate). The enzyme has no detectable hydrolytic action on these compounds.

[2] Cbz, carbobenzoxy.

With the oxidized B chain of insulin as the substrate (5), it was found that Proteinase I resembles pepsin in its action. Cathepsin A of beef spleen (9, 10) also resembles pepsin in its specificity; however, both of these enzymes readily hydrolyze Cbz-L-glutamyl-L-tyrosine whereas Proteinase I does not.

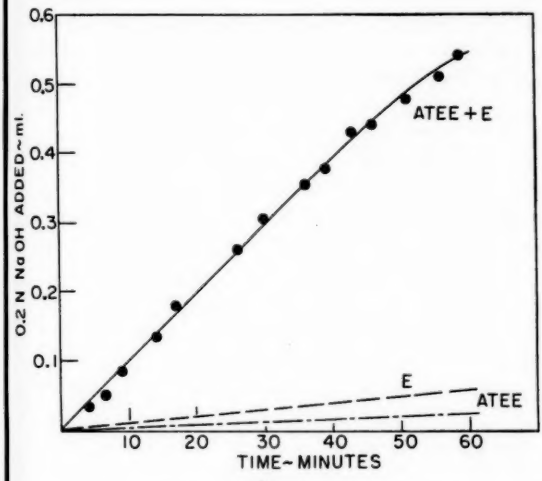


FIG. 4

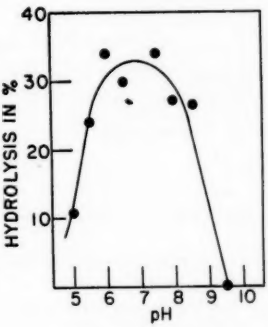


FIG. 5

FIG. 4. Hydrolysis of 0.05 M ATEE by Proteinase II (8.4 mg. of protein N per ml.) at pH 7.5. NaOH at 0.2 N was used to maintain constant pH, and the amount consumed in a given time multiplied by 80 represents hydrolysis of the ester in per cent. The enzyme preparation (*E*) was made by extracting 220 gm. of frozen beef lung in 110 ml. of cold water in a Waring blendor and centrifuging the insoluble débris. No buffer was used. The ester control was run in a solution of human γ-globulin in physiological saline.

FIG. 5. Effect of pH on the hydrolysis of 0.05 M ATEE by Proteinase II at 8.4 mg. of protein N per ml. The ordinate gives the hydrolysis, in per cent, after correction for non-enzymatic hydrolysis and autolysis of the enzyme preparation.

Properties of Proteinase II

No attempt has been made as yet to purify this enzyme, and the following studies were made with crude, centrifuged, saline extracts of bovine lung. The enzyme is relatively unstable. Only 5 per cent of the activity is recovered after 24 days at 5° in the presence of 1:50,000 merthiolate. Fe^{++}, Mn^{++}, and Zn^{++} do not influence the activity. Phosphate at 0.2 M inhibits the enzyme 66 per cent; a similar effect on a proteinase of pituitary has been noted earlier (11).

Proteinase II was tested for its ability to hydrolyze several synthetic

substrates. After 18 hours *N*-acetyl-L-tyrosinamide, a substrate for chymotrypsin, was hydrolyzed about 20 per cent by extracts of both rat (at 0.88 mg. of protein N per ml.) and beef lung (at 0.59 mg. of protein N per ml.). Other substrates were not hydrolyzed appreciably; *e.g.*, benzoyl-L-argininamide, Cbz-L-glutamyl-L-tyrosine, and Cbz-L-isoglutamine. It has been shown (12) that chymotrypsin will hydrolyze certain esters far more rapidly than the corresponding amides. *N*-Acetyl-L-tyrosine ethyl ester (ATEE) was, therefore, tested as a substrate for Proteinase II. The method of Schwert *et al.* (13) was used to measure the rate of ester hydrolysis.

TABLE III

Effect of Inhibitors on Proteinase II

A water extract of beef lung was incubated with the inhibitor at pH 8 for 30 minutes at 33° before assay.

Inhibitor	Inhibition	
	Hemoglobin as substrate	ATEE as substrate
	per cent	*per cent*
Diisopropyl fluorophosphate (0.0004 M)	0	49
Iodoacetamide (0.002 M)	50	34
p-Chloromercuribenzoate (0.0002 M)	50	0
Versene (0.02 M)	75	5
Phosphate (0.2 M)	66	16

Fig. 4 shows that the initial hydrolysis of ATEE is essentially linear with time. Controls for non-enzymatic hydrolysis of the ester and for autolysis of the enzyme preparation are also given. The hydrolysis of ATEE is optimal near pH 7 (Fig. 5).

Table III lists the effect of certain inhibitors on the activity of Proteinase II. With hemoglobin as substrate, the enzyme concentration was 1.5 mg. of protein N per ml. of reaction mixture at pH 8.5; with ATEE as substrate, 8.4 mg. of protein N and pH 8.0 were used. Although the difference in protein concentration may influence the degree of inhibition, this cannot be responsible for the differences in behavior with the two substrates. DFP is a strong inhibitor of the esterase action and has no effect on hemoglobin hydrolysis. Similarly, the small effect of Versene and phosphate on the esterase action is noteworthy. These results suggest that "Proteinase II" includes more than one enzyme and that the proteinase mainly responsible for the hydrolysis of hemoglobin is distinct from the one which attacks the ester.

DISCUSSION

The above results show that there are two pH regions of proteinase action in extracts of beef and rat lung. Proteinases having similar pH optima have been described from many other tissues; *e.g.*, spleen (14), brain (15, 16), pituitary (11), erythrocytes (17), and polymorphonuclear cells (18). Mononuclear phagocytes (18, 19), muscle (20), and thyroid (21) have been found to possess only proteinases optimally active in the acid pH range.

The limited information presently available concerning the chemical nature and specificity of all these enzymes does not permit any firm conclusions regarding the relationships among them. It should be noted, however, that some cells and tissues, *e.g.* pituitary (11), erythrocytes (17), and lung, possess proteinases which do not attack the simple synthetic compounds which are substrates for the proteinases of spleen, liver, and kidney (9, 10). These findings suggest that a considerable variation exists among animal tissues and that some may possess distinct proteinases limited to one or a few tissues.

In contrast to the proteinases, it should be emphasized that certain peptidases, *e.g.* leucine aminopeptidase, prolidase, tripeptidase, etc., appear to be the same in lung as in all other animal tissues examined so far.

SUMMARY

1. Rat and beef lung contain leucine aminopeptidase, tripeptidase, glycylglycine dipeptidase, prolidase, and iminodipeptidase.

2. The hydrolytic action of lung extracts on hemoglobin shows two regions of optimal activity, at pH 4.0 (Proteinase I) and at pH 8.4 (Proteinase II).

3. A method of purifying beef lung Proteinase I about 150-fold is described. This preparation is about one-tenth as active as crystalline trypsin on hemoglobin. Proteinase I is inhibited by *p*-chloromercuribenzoate and by iodoacetamide; this suggests that the enzyme has an essential sulfhydryl group. No synthetic substrate has been found for Proteinase I.

4. Crude Proteinase II hydrolyzes *N*-acetyl-L-tyrosine ethyl ester with an optimal action at pH 7. Comparison of the effect of various inhibitors on the hydrolysis of hemoglobin and of the ester substrate suggests that more than one enzyme is concerned in the activity of Proteinase II.

BIBLIOGRAPHY

1. Fruton, J. S., *J. Biol. Chem.*, **166**, 721 (1946).
2. Weiss, C., and Halliday, N., *J. Immunol.*, **49**, 251 (1944).
3. Nye, R. N., *J. Exp. Med.*, **35**, 153 (1922).
4. Weiss, C., *Arch. Path.*, **33**, 182 (1942).

5. Dannenberg, A. M., Jr., and Smith, E. L., *J. Biol. Chem.*, **215,** 55 (1955).
6. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183,** 32 (1929).
7. Smith, E. L., *Advances in Enzymol.*, **12,** 191 (1951).
8. Kunitz, M., *J. Gen. Physiol.*, **30,** 291 (1947).
9. Tallan, H. H., Jones, M. E., and Fruton, J. S., *J. Biol. Chem.*, **194,** 793 (1952).
10. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130,** 19 (1939).
11. Adams, E., and Smith, E. L., *J. Biol. Chem.*, **191,** 651 (1951).
12. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177,** 793 (1949).
13. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172,** 221 (1948).
14. Hedin, S. G., *J. Physiol.*, **30,** 155 (1904).
15. Kies, M. W., and Schwimmer, S., *J. Biol. Chem.*, **145,** 685 (1942).
16. Ansell, G. B., and Richter, D., *Biochim. et biophys. acta*, **13,** 87, 92 (1954).
17. Morrison, W. L., and Neurath, H., *J. Biol. Chem.*, **200,** 39 (1953).
18. Weiss, C., and Czarnetsky, E. J., *Arch. Path.*, **20,** 233 (1935).
19. Opie, E. L., *J. Exp. Med.*, **8,** 410 (1906).
20. Snoke, J. E., and Neurath, H., *J. Biol. Chem.*, **187,** 127 (1950).
21. De Robertis, E., *Anat. Rec.*, **80,** 219 (1941).

ACTION OF PROTEINASE I OF BOVINE LUNG. HYDROLYSIS OF THE OXIDIZED B CHAIN OF INSULIN; POLYMER FORMATION FROM AMINO ACID ESTERS*

By ARTHUR M. DANNENBERG, Jr.,† and EMIL L. SMITH

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

(Received for publication, November 8, 1954)

The first studies (1) on the specificity of proteinases were performed on simple synthetic compounds. Such investigations had to be conducted for the most part on an empirical basis, inasmuch as methods were not available for determining the sites of action of the enzyme on proteins or larger peptides. The work of Sanger and coworkers (2, 3) on the action of proteolytic enzymes on the oxidized A and B chains of insulin has provided another method of studying these enzymes. These investigators confirmed previous knowledge of the specificity of pepsin, chymotrypsin, and trypsin, and discovered unrecognized sites of action of chymotrypsin and pepsin.

With Proteinase I of bovine lung, we were unable to find a suitable synthetic substrate (4), and we have, therefore, investigated the action of the enzyme on the oxidized B chain of insulin. Proteinase I acts on many of the same loci as does crystalline pepsin, although Proteinase I has no detectable action on compounds like acetyl-L-phenylalanyl-L-phenylalanine (4) which are readily hydrolyzed by pepsin (5). It is likely that other, as yet unrecognized, features are required in low molecular weight compounds in order to be suitable as substrates for the hydrolytic action of Proteinase I. While our study was in progress, Tuppy (6) determined the specificity of a proteinase from *Bacillus subtilis* by its action on the oxidized B chain of insulin. These methods appear to provide a new and powerful tool for determining the specificity of proteinases.

Proteinase I catalyzes the formation of polypeptide derivatives from amino acid amides and esters. In the formation of the amino acid polymers, presumably by a transfer mechanism, the enzyme exhibits a specificity similar to that of its hydrolytic action, inasmuch as its activity is greatest when aromatic, large aliphatic, and glutamic acid residues are involved. Fruton and coworkers (7–9) have described a number of transfer (transpeptidation) reactions catalyzed by proteinases, notably the action of ca-

* This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service.

† Postdoctoral Fellow of the National Tuberculosis Association, administered by the National Research Council.

thepsin C of beef spleen which acts on dipeptide amides or esters to form insoluble polymeric compounds. In contrast to Proteinase I, cathepsin C has no action on amino acid esters or amides (8, 9). However, chymotrypsin catalyzes transfer reactions with amino acid esters (10, 11).

Digestion of Oxidized B Chain of Insulin

The oxidized B chain of insulin[1] was prepared from crystalline swine insulin[2] by the method of Sanger (12, 13). Proteinase I was purified about 150-fold from bovine lung, as already described (4). Proteinase I (0.027 mg. of protein N per ml. of test solution) and the B chain (5.0 mg. per ml. of test solution) were incubated at 39° for 4 hours at pH 4.0 in acetate buffer of 0.1 ionic strength. The enzymatic digestion was stopped by the addition of enough solid $NaHCO_3$ to bring the pH to 8.3. A control sample was prepared which had the same composition as the experimental sample, but was not incubated. The B chain without Proteinase I was also kept as a control at pH 4.0.

With a ninhydrin method (14) it was found that between three and four amino groups were liberated per mole of B chain in 4 hours. The digestion was also performed with higher concentrations of enzyme and for longer periods of incubation. Although these conditions gave more amino groups, as estimated by ninhydrin, control samples without the B chain showed significant autolysis of the enzyme. With the enzyme concentration given above and 4 hours hydrolysis, the concentration of amino end-groups arising from autolysis of the enzyme preparation was insignificant.

Determination of N-Terminal Amino Acids—The enzymatic digest of the B chain was treated with 1,2,4-fluorodinitrobenzene (FDNB) (15) and the dinitrophenylated peptides (DNP peptides) were separated as a group in the usual manner. These were hydrolyzed in 6 N HCl in a sealed glass tube at 105° for 16 hours. Separate experiments were run to show the absence of DNP-proline and DNP-glycine as *N*-terminal residues of peptides.

After hydrolysis, the solution was diluted with water and the DNP-amino acids were extracted into ether. The aqueous layer contained only ε-DNP-lysine (16). The ether layer, worked up by methods described earlier (15, 17), contained the DNP derivatives of the *N*-terminal amino acids of the peptides resulting from the enzymatic hydrolysis, and DNP-phenylalanine, the *N*-terminal residue of the B chain (12). The DNP amino acids were separated and identified on paper chromatograms with

[1] We are indebted to Dr. E. O. P. Thompson for his advice and help in the preparation of the oxidized B chain of insulin and in the use of the chromatographic techniques.

[2] We are indebted to Mr. Tillman D. Gerlough of E. R. Squibb and Sons for these samples.

buffered tertiary amyl alcohol (18), phosphate buffer (19), and toluene-pyridine-chlorohydrin (20). With tertiary amyl alcohol, spots were obtained in the region of DNP-tyrosine and DNP-leucine. These were eluted with 1 per cent $NaHCO_3$, acidified, extracted into ether, and run on paper in the toluene-pyridine-chlorohydrin system for resolution.

Partition chromatography on buffered Celite No. 545 columns (17, 21) was also used. Quantitative determinations were made by dissolving the DNP-amino acids obtained from the columns in 1 per cent aqueous $NaHCO_3$ and reading their absorption at 350 mμ with the Beckman spectrophotometer.

The amino acids were regenerated (22) from the DNP-leucine and the DNP-phenylalanine bands obtained from the Celite columns. The leucine

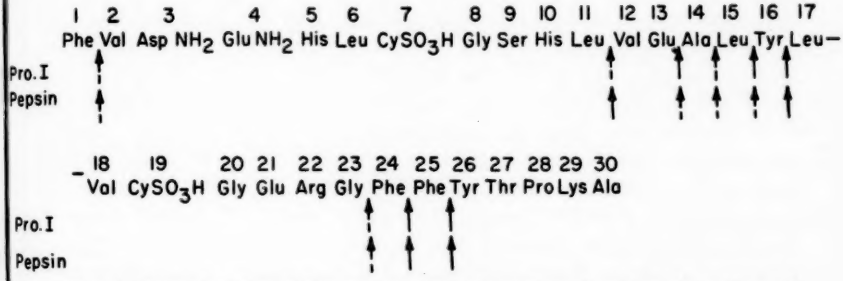

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Phe Val Asp NH2 Glu NH2 His Leu CySO3H Gly Ser His Leu Val Glu Ala Leu Tyr Leu
Pro. I
Pepsin
18 19 20 21 22 23 24 25 26 27 28 29 30
Val CySO3H Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala
Pro. I
Pepsin

FIG. 1. Sites of action of Proteinase I (Pro. I) and pepsin (2) on the oxidized B chain of insulin. The abbreviations for the amino acid residues are those used by Sanger and Tuppy (2). The arrow indicates a major site of action; the broken arrow indicates other bonds split by pepsin and probably by Proteinase I.

and the phenylalanine liberated by this procedure were identified on a paper chromatogram with butanol-acetic acid-water (5:1:4) as the solvent (23).

DNP-alanine, DNP-phenylalanine, di-DNP-tyrosine, DNP-valine, and DNP-leucine were identified in the experimental sample. Only DNP-phenylalanine was found in significant quantity in the controls. There was about twice the amount of DNP-phenylalanine in the experimental sample as in the controls, as judged from identical aliquots. From these results and the known structure of the B chain, the sites of action of Proteinase I may be assigned as shown in Fig. 1. In order to confirm these results, some of the peptides resulting from the action of Proteinase I on oxidized B chain were also studied.

N-Terminal Sequences of Liberated Peptides—Oxidized B chain was digested by Proteinase I and treated with FDNB as already described. The resulting DNP peptides were partially hydrolyzed in 12 N HCl at 37° for

5 days. Water was added and the DNP derivatives were extracted into ethyl acetate. This solution was washed twice with 1 N HCl and repeatedly concentrated to dryness *in vacuo*. The residue was dissolved in wet chloroform and chromatographed on buffered Celite columns (17).

On the Celite column of pH 6.5, two bands with R values[3] of approximately 0.10 and 0.02 were isolated; these were shown to be DNP-alanylleucine and DNP-alanine, respectively, by the following procedure. After acidification, the DNP derivatives were extracted into ether and taken to dryness *in vacuo*. After complete hydrolysis, DNP-alanine was identified as the *N*-terminal amino acid in both samples. Leucine was identified in the aqueous phase from the band with $R = 0.10$. No amino acid was found in the aqueous phase from the band with $R = 0.02$. The enzyme must have hydrolyzed the glutamylalanyl bond of the B chain; this is the only locus from which alanylleucine could be liberated from the B chain.

On the Celite column of pH 7.1 with wet chloroform as the solvent, a broad band ($R = 0.2$ to 0.4) was isolated. This fraction was hydrolyzed completely and worked up in the usual way. DNP-phenylalanine and di-DNP-tyrosine were found in approximately a 3:1 ratio. Valine and leucine were found in the aqueous phase, also in a 3:1 ratio. From the known sequence of the B chain (Fig. 1) and the 3:1 ratio in each group, it seems highly probable that DNP-phenylalanylvaline and di-DNP-tyrosylleucine are the DNP peptides present in the broad band ($R = 0.2$ to 0.4). Phenylalanylvaline is the *N*-terminal sequence of the B chain. The isolation of di-DNP-tyrosylleucine signifies that Proteinase I hydrolyzed the leucyltyrosyl bond of the sequence alanylleucyltyrosylleucyl.

No other DNP peptide sequences were found; however, free DNP-phenylalanine was isolated in an amount greater than might be expected if only the *N*-terminal phenylalanine were the source of this compound. This supports other evidence that the sequence phenylalanylphenylalanyltyrosine is hydrolyzed at both bonds.

Isolation of Peptide—After an incubation period of 3 hours, 1.5 ml. were removed from the enzymatic digest of the B chain. This was brought to pH 9.5 with 1 N NaOH. Approximately 0.6 ml. was placed in a narrow band on the center of a sheet of Whatman No. 3 paper. Ionophoresis (24) was performed at 500 volts for 5 hours in ammonium acetate buffer (0.1 M, pH 9.4) in a closed tank at room temperature. Strips of 1 cm. width were cut from each side and from the center of the dried paper, and these were sprayed with 0.1 per cent ninhydrin in 95 per cent ethanol. Similar strips were sprayed with the Pauly reagent for histidine.

A 1 cm. band, strongly positive to the ninhydrin reagent, had migrated

[3] R is the rate of movement of the band on the column compared to that of the solvent above the column.

2 cm. towards the negative pole. This same band was only faintly positive to the Pauly reagent. From the formula of the B chain and the sites of Proteinase I action already identified, it seemed likely that this band contained a lysine peptide. Since the Pauly test was faintly positive, a small amount of histidine was also present. This band was cut from the paper and eluted with water (25). After concentration *in vacuo*, the material was placed on Whatman No. 3 paper for chromatography with butanol-acetic acid.

After 16 hours the chromatogram was dried and sprayed with 0.025 per cent ninhydrin in 95 per cent ethanol. A yellow spot and a large purple spot appeared. The latter was eluted with water and hydrolyzed completely. After repeated concentration *in vacuo*, the residue was chromatographed with phenol-0.3 per cent aqueous ammonia (26) as the solvent for the first dimension and butanol-acetic acid for the second dimension. Only tyrosine, threonine, proline, lysine, and alanine were present. These are the amino acids of the *C*-terminal sequence of the B chain (2, 25). Since no phenylalanine was detected, Proteinase I must hydrolyze the oxidized B chain between phenylalanine and tyrosine.

Polymer Formation Catalyzed by Proteinase I

When purified Proteinase I was incubated near pH 7 with the esters or amides of L-leucine or L-phenylalanine, the formation of precipitates was observed. It was noted that incubation of the esters led to more rapid precipitation than the amides. With the exception of some comparative observations reported below, most of the studies were performed with L-phenylalanine methyl ester.

Methods—The amount of polymer formation could be estimated visually as a convenient qualitative method. Quantitative measurements of the disappearance of α-amino groups were made by a ninhydrin method (14). L-Phenylalanine ester at 0.05 M was incubated with Proteinase I (0.04 mg. of protein N per ml.) in the presence of 0.004 M cysteine. At appropriate intervals 0.1 ml. aliquots were removed and added to 2.0 ml. of 0.2 M citrate buffer at pH 5.0; 0.1 ml. of this solution was then added to the ninhydrin reagent and the procedure of Moore and Stein (14) was followed. The ninhydrin colors were read at 570 mμ with a Coleman junior spectrophotometer. Control samples without substrate and without enzyme were run simultaneously.

As indicated later, the phenylalanine polymer appears to have an average size of 6 residues. Therefore, a decrease of 83 per cent in ninhydrin color of the experimental sample would represent 100 per cent synthesis on the assumption that the terminal amino group of the polypeptide has the same color value as an amino group in phenylalanine ester. Although this

may be only an approximation, it is adequate for the comparative studies reported below.

Effect of Time and pH—The amount of polymer formation is shown in Fig. 2 as a function of time for several pH values. At acid pH values, the extent of polymerization increases to at least 20 hours, whereas at alkaline

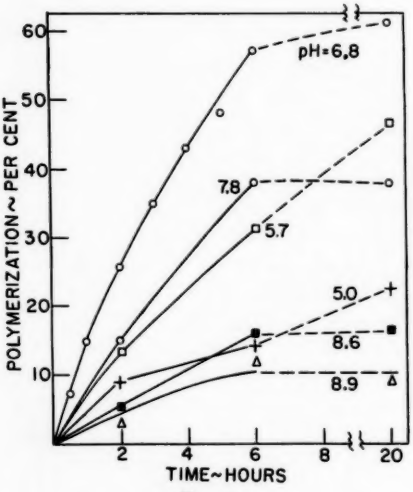


FIG. 2

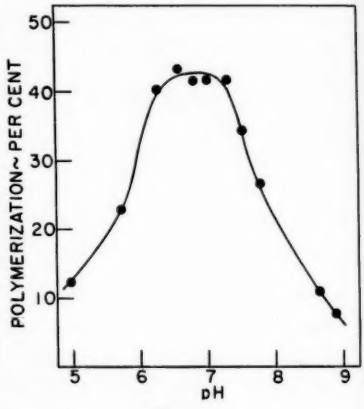


FIG. 3

FIG. 2. Formation of polyphenylalanine from L-phenylalanine methyl ester as a function of time at different pH values. The substrate at 0.05 M was incubated at 38° in the presence of 0.004 M cysteine. The concentration of Proteinase I was 0.04 mg. of protein N per ml. The amount of polymer formation was calculated from the decrease of α-amino groups estimated by the ninhydrin method. The following buffers at 0.2 M were used: pH 5 and 5.7, citrate; pH 6.8, phosphate; pH 7.8 and above, Tris (tris(hydroxymethyl)aminomethane).

FIG. 3. Effect of pH on the formation of polyphenylalanine by Proteinase I. The extent of polymer formation was estimated at 4 hours by the method described in Fig. 2. pH was controlled by the following buffers at 0.2 M: pH 5 to 6, citrate; pH 6.2 to 6.8, phosphate; above pH 6.8, Tris.

pH values the reaction ceases after about 6 hours. This may be due, in part, to the saponification of the ester at the high pH values which effectively lowers the substrate concentration. The total extent of synthesis is about 60 per cent at pH 6.8, which is high when it is considered that hydrolysis of the ester may also be occurring. Fruton *et al.* (8) have found that cathepsin C is removed from solution by precipitated polymer; this factor may also be involved in the case of Proteinase I.

Fig. 3 shows that polymer formation is optimal at pH 6.5 to 7.2.

Specificity—Fifteen amino acid esters were used as substrates with Proteinase I at pH 6.8 to 7.0; the results are given in Table I. The results obtained by the ninhydrin method have been computed on the basis of a polymer containing 6 residues. Although this may not be valid for all the compounds, the values may be regarded as reasonable comparative estimates.

TABLE I

Polymerase Activity of Proteinase I with Amino Acid Esters

The amino acid esters at 0.05 M (0.1 M for the racemic compound) were incubated at 38° for 4 hours with purified Proteinase I at a concentration of 0.04 mg. of protein N per ml. The mixtures contained 0.004 M cysteine and 0.2 M phosphate buffer at pH 6.8. The amount of ester polymerized was estimated by the decrease of ninhydrin color as described in the text. The amount of precipitation was estimated visually.

Substrate	Amount of polymerization	Pptn.	Substrate	Amount of polymerization	Pptn.
	per cent			*per cent*	
L-Phenylalanine methyl ester	43	++++	L-Alanine methyl ester	6	–
L-Glutamic acid diethyl ester	38	++	L-Aspartic acid diethyl ester	4	–
L-Tryptophan methyl ester	29	++++	L-Lysine methyl ester L-Histidine methyl ester	2	–
L-Tyrosine ethyl ester*	30	++	DL-Serine methyl ester	0	–
L-Leucine " "	27	+++	L-Proline benzyl ester‡	0	–
L-Valine methyl ester†	12	+	L-Hydroxyproline methyl ester‡	0	–
L-Arginine ethyl "	11	±			
L-Glycine " "	10	–			

* This preparation set to a clear gel.

† Definite precipitation only after 6 hours.

‡ The ninhydrin colors were read at 440 mμ instead of at 570 mμ.

Maximal polymerization occurred with the aromatic amino acid esters. The precipitate of polytryptophan appeared most quickly, but, after 4 hours, was approximately equal to that of polyphenylalanine. The quantity of polytyrosine was difficult to estimate visually, since a clear, viscid gel was formed.

Control samples of glutamic acid diester without enzyme gave some decrease of free amino groups in 4 hours; this may be attributed to the formation of pyrrolidonecarboxylic acid which is known to occur under these conditions. In calculating the extent of the glutamic polymer formation, correction was made for the decrease of ninhydrin color of the control. Nevertheless, the amount of polymer formed appeared to be less by visual

inspection than by ninhydrin estimation. One possible explanation is that some hydrolysis of the γ-ester linkages may have occurred; this would be expected to increase the solubility of the polymers.

The formation of a precipitate from valine ester was slow and was definite only after 6 hours. The remaining compounds in Table I showed no precipitation after 6 hours, although in some cases there was a small decrease in ninhydrin color which may not have been indicative of polymer formation.

Activation and Inhibition—Preliminary tests showed that cysteine increased the velocity of polymer formation, and this substance was used as an activator in the studies described above. With 2 hours incubation, 0.004 M cysteine doubled the rate of formation of polymer from phenylalanine ester as judged by the ninhydrin method. In some experiments, Versene (ethylenediaminetetraacetate) also increased the rate of polymer formation. *p*-Chloromercuribenzoate (0.0002 M) and iodoacetamide (0.002 M) produced complete inhibition of the polymerization of phenylalanine ester as judged both by the absence of visible turbidity and by the constancy of ninhydrin color. Diisopropyl fluorophosphate at 0.00027 M had no effect on the reaction whatsoever.

All the above activators and inhibitors were mixed with the enzyme at pH 5 to 6 and kept at room temperature for 30 to 60 minutes before the mixture was added to the substrate. The samples were assayed at pH 6.8 in the presence of 0.2 M phosphate buffer.

Characterization of Polyphenylalanine—By analogy with the reactions studied by Fruton and coworkers (7–9), it may be anticipated that the polymerization of amino acid esters and amides produced by Proteinase I yields the corresponding polypeptide derivative or polypeptide, or a mixture of the two. Some experiments were performed to ascertain the size of the compounds formed.

Phenylalanine ester (0.05 M) was incubated at 38° for 22 hours with Proteinase I (0.05 mg. of protein N per ml.) in 40 ml. of solution containing 0.0025 M cysteine and 0.1 M phosphate buffer at pH 6.9. At the end of the reaction the pH was 6.5; the change may be due to some hydrolysis of the ester. The white precipitate was centrifuged, washed four times with 40 ml. portions of water, and dried *in vacuo* over P_2O_5. The yield was 30 per cent as calculated from the initial concentration of phenylalanine ester.

Approximate estimates of the chain length were obtained by comparing the ninhydrin colors derived from the phenylalanine and leucine polymers with that of standard solutions of the amino acids with 66 per cent phenol as the solvent. The polymers gave colors equivalent to one amino group for 10.5 phenylalanine residues and for 10.0 leucine residues. It should be noted, however, that the ninhydrin color derived from polypeptides is fre-

XUM

quently less than that expected for an equivalent amount of amino groups on free amino acids.

A further estimate was made by the FDNB method (15). DNP-polyphenylalanine was prepared and then hydrolyzed in 6 N HCl for 17 hours at 110°. Ether solutions of the DNP derivatives were chromatographed on Celite columns (17) or on paper (20). In both cases the principal compound found was DNP-phenylalanine, but some DNP-phenylalanine peptides were also present, presumably because of the difficulty of obtaining complete hydrolysis of the very insoluble DNP polypeptide.

The DNP peptides were estimated spectrophotometrically with DNP-phenylalanine inasmuch as absorption of the DNP group is equivalent in peptides and amino acids (27). The results for the separation on columns gave one DNP-phenylalanine group per 6.7 residues, and, on paper, one group per 5.6 residues; the average is 6.1 residues. These results are corrected for an average destruction during hydrolysis of 33 per cent of DNP-phenylalanine, as determined in parallel control experiments.

Synthesis of Polymeric Peptides by Extract of Mononuclear Cells—As previously indicated (4), it is of some importance to determine the origin of Proteinase I. Presumably this enzyme could be derived from the mononuclear phagocytes present in this tissue. Preparations rich in mononuclear cells were obtained by the following procedure.

Two mature, female albino rabbits were injected intraperitoneally with 40 ml. of sterile mineral oil (U. S. P.). After 6 days they were sacrificed by air embolism and the peritoneal cavities were washed with 200 ml. of physiological saline solution containing 0.4 per cent sodium citrate. The cells were collected by centrifugation and washed three times with 40 ml. portions of the citrate-saline solution. A sample of the precipitate was smeared onto cover-slips, stained with Wright's stain, and examined under the microscope. Only mononuclear cells and erythrocytes were present; no granulocytes were seen. The mononuclear cells were of both the large and the small variety, the latter being typical lymphocytes. The red cells represented about two-thirds the area of the smear as well as about two-thirds the packed cell volume after centrifugation. Because of the presence of erythrocytes in the exudate, a sample of rabbit heart blood was collected and prepared by the procedure given above. Both the exudate cells and the erythrocytes were suspended in saline and then treated with acetone at −30° to prepare acetone-dried powders in the usual manner.

A sample (22 mg.) of the powdered mononuclear cells was ground in 9.0 ml. of water and centrifuged. The dried erythrocytes were treated similarly as a control. The extracts were assayed at 39° for 17 hours with 0.1 M L-phenylalanine methyl ester and 0.05 M L-glutamic acid diethyl ester as substrates in the presence of 0.004 M cysteine with 0.2 M phosphate buf-

fer at pH 6.8. The final concentration in each preparation was 0.07 mg. of protein N per ml. Control samples of the esters without extracts were run, and of the extracts without substrates under the same conditions as the experimental samples.

A precipitate was present in the experimental sample from mononuclear cells containing the phenylalanine ester. None was present in the control samples or the samples with erythrocyte extracts. The ninhydrin method showed that 10 per cent of the phenylalanine ester had been polymerized in the experimental sample and none in the control. The extract of mononuclear cells polymerized the glutamic acid ester to almost the same degree, as judged by the decrease in ninhydrin color.

DISCUSSION

Proteinase I of beef lung has a specificity similar to pepsin (Fig. 1) in that it hydrolyzes the phenylalanyltyrosyl, phenylalanylphenylalanyl, leucyltyrosyl, and glutamylalanyl bonds of the oxidized B chain of insulin. As shown in Fig. 1, pepsin splits certain bonds of the oxidized B chain that contain valine and leucine. These amino acids have also been detected as the *N*-terminal residues of peptides liberated by Proteinase I; however, no experiments have been performed to determine which valyl and leucyl bonds are involved.

The studies of Sanger *et al.* (2, 3, 13, 25) were performed on bovine insulin. Assuming that the B chains of the two species do not differ in any fundamental way, we have used porcine insulin. The two proteins differ in that porcine insulin contains a threonine in place of an alanine residue, and an isoleucine instead of a valine (28). The threonine-alanine difference is in the A chain (29). The location of the isoleucine-valine difference is unknown, although our characterization of DNP-phenylalanylvaline indicates that the difference is not at the *N*-terminal end of the B chain.

The hydrolytic action of Proteinase I on hemoglobin is partially inhibited at pH 4 by the sulfhydryl reagents, *p*-chloromercuribenzoate and iodoacetamide (4). At pH 7 the polymerase action is completely inhibited by the same substances. This is due presumably to the difference in concentration at the two pH values of the susceptible form of the sulfhydryl group, which is undoubtedly RS^- rather than RSH.

The difference in pH optima for the two actions of the enzyme is striking. For hydrolysis, the optimum is near pH 4 (4), whereas, for polymerization of phenylalanine ester, the optimum is near pH 7. Similar differences have been noted for other proteinases which manifest transferase activity (7, 9), and have been ascribed to the difference in effective concentration of the agent ($R\text{-}NH_2$) which displaces the ester grouping.

It is noteworthy that the specificity of Proteinase I, as determined by

its action on the B chain and by its polymerase (or transferase) activity on amino acid esters, is essentially similar. For both types of action, residues involving aromatic, large aliphatic side chains and glutamic acid are most favorable. This finding, as well as a similar sensitivity to sulfhydryl reagents, suggests that both types of action are due to the same proteinase.

Crude extracts of rabbit mononuclear cells show a polymerase activity towards phenylalanine ester and glutamic acid diester. This suggests that Proteinase I of lung may be derived from such cells.

SUMMARY

1. The specificity of purified Proteinase I obtained from bovine lung has been determined by using the oxidized B chain of insulin as a substrate and analyzing the split-products liberated. The results indicate that Proteinase I has a specificity similar to crystalline pepsin.

2. Proteinase I catalyzes the synthesis of polypeptides from certain amino acid esters and amides. The optimum for the synthesis of polyphenylalanine from L-phenylalanine methyl ester is near pH 6.8. The rate of formation of polymeric peptides is activated by cysteine and inhibited by iodoacetate and *p*-chloromercuribenzoate. These results indicate that the enzyme has an essential sulfhydryl group, and suggest that the synthetic and hydrolytic actions are due to the same enzyme.

3. The specificity for the polymerization reaction parallels the hydrolytic action of the enzyme on the oxidized B chain of insulin; *i.e.*, there is a preference for the aromatic and long chain aliphatic amino acids as well as for glutamic acid derivatives.

4. An extract of rabbit mononuclear phagocytic cells exhibits polymerase action with amino acid esters under the same conditions as Proteinase I; this finding suggests that these cells may be the source of the enzyme.

BIBLIOGRAPHY

1. Bergmann, M., and Fruton, J. S., *Advances in Enzymol.*, **1**, 63 (1941).
2. Sanger, F., and Tuppy, H., *Biochem. J.*, **49**, 481 (1951).
3. Sanger, F., and Thompson, E. O. P., *Biochem. J.*, **53**, 366 (1953).
4. Dannenberg, A. M., Jr., and Smith, E. L., *J. Biol. Chem.*, **215**, 45 (1955).
5. Baker, L. E., *J. Biol. Chem.*, **193**, 809 (1951).
6. Tuppy, H., *Monatsch. Chem.*, **84**, 996 (1953).
7. Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S., *J. Biol. Chem.*, **195**, 645 (1952).
8. Fruton, J. S., Hearn, W. R., Ingram, V. M., Wiggans, D. S., and Winitz, M., *J. Biol. Chem.*, **204**, 891 (1953).
9. Wiggans, D. S., Winitz, M., and Fruton, J. S., *Yale J. Biol. and Med.*, **27**, 11 (1954).
10. Brenner, M., Müller, H. R., and Pfister, R. W., *Helv. chim. acta*, **33**, 568 (1950).
11. Tauber, H., *J. Am. Chem. Soc.*, **74**, 847 (1952).

XUM

12. Sanger, F., *Biochem. J.*, **44,** 126 (1949).
13. Sanger, F., and Thompson, E. O. P., *Biochem. J.*, **53,** 353 (1953).
14. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176,** 367 (1948).
15. Sanger, F., *Biochem. J.*, **39,** 507 (1945).
16. Bailey, K., *Biochem. J.*, **49,** 23 (1951).
17. Thompson, E. O. P., *J. Biol. Chem.*, **207,** 563 (1954).
18. Blackburn, S., and Lowther, A. G., *Biochem. J.*, **48,** 126 (1951).
19. Levy, A. L., and Chung, D., *Nature*, **174,** 126 (1954).
20. Biserte, G., and Osteux, R., *Bull. soc. chim. biol.*, **33,** 50 (1951).
21. Perrone, J. C., *Nature*, **167,** 513 (1951).
22. Mills, G. L., *Nature*, **165,** 403 (1950).
23. Partridge, S. M., *Biochem. J.*, **42,** 238 (1948).
24. Flynn, F. V., and de Mayo, P., *Lancet*, **2,** 235 (1951).
25. Sanger, F., and Tuppy, H., *Biochem. J.*, **49,** 463 (1951).
26. Draper, O. J., and Pollard, A. L., *Science*, **109,** 448 (1949).
27. Sanger, F., *Biochem. J.*, **45,** 563 (1949).
28. Harfenist, E. J., *J. Am. Chem. Soc.*, **75,** 5528 (1953).
29. Sanger, F., *Nature*, **164,** 529 (1949).

ISOLATION AND PROPERTIES OF A CRYSTALLINE MERCURY DERIVATIVE OF A LYSOZYME FROM PAPAYA LATEX*

By EMIL L. SMITH, J. R. KIMMEL,† DOUGLAS M. BROWN, AND E. O. P. THOMPSON

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

(Received for publication, November 8, 1954)

In his survey of lytic activity in various biological materials, Fleming (1, 2) noted that some plant tissues contain lysozyme. Meyer *et al.* (3) observed later that crude preparations of the plant proteinases, ficin and papain, are very rich in lysozyme activity. We wish to report in this paper the isolation of a lysozyme obtained as a crystalline mercury derivative from commercial preparations of dried papaya latex. The enzymatic behavior, amino acid composition, and some physical properties will be described and compared with the lysozymes obtained from animal tissues.

Our identification of papaya lysozyme is of interest inasmuch as the protein was crystallized during attempts to isolate other proteinases from commercial latex after the successful preparation of crystalline papain (4). Since the recrystallized protein was devoid of proteinase activity, a survey was made of its physical and chemical properties. A study of the amino acid composition showed a relatively low content of histidine and glutamic acid, and relatively large amounts of arginine and tryptophan. These findings, in conjunction with a basic isoelectric point (pH 10.5) and a relatively low molecular weight near 25,000, suggested an examination for lysozyme activity with *Sarcina lutea* as the test organism. The new enzyme proved to have an activity on a molar basis about one-fifth that of crystalline lysozyme from egg white. It would appear that this is one of the rare instances in which a protein has been isolated and its enzymatic activity identified from its chemical and physical properties.

Method of Assay

Assays of lysozyme activity were performed essentially by the turbidimetric method of Dickman and Proctor (5). Portions of a frozen suspen-

* This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service, and from the Rockefeller Foundation.

† Postdoctoral Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

XUM

sion of *S. lutea*[1] were thawed and diluted with water until 1 ml. of this suspension, mixed with 3 ml. of water and 0.4 ml. of 4 M NaOH, showed an apparent optical density at 440 mμ equal to approximately 0.7. Vigorous stirring of this suspension at the time of preparation was necessary to insure the absence of large particles. The 5 ml. assay flasks contained 1.25 ml. of the suspension of the organism, 2.5 ml. of 0.1 M acetate buffer at pH 4.65, and, if necessary, 0.5 ml. of 0.05 M cysteine. This mixture was incubated for 10 minutes at 39°, 0.5 ml. of enzyme solution was added, and the mixture was diluted to 5 ml. At appropriate intervals, 1 ml. aliquots of the reaction mixture were transferred to 10 × 75 mm. colorimeter tubes, 0.1 ml. of 4 M NaOH was added, and, after 30 minutes at room temperature, the apparent optical density (A_t) at 440 mμ was measured in a Coleman junior spectrophotometer. The absorbancy of a completely lyzed bacterial suspension (A_f) was determined in the same manner. Complete lysis was achieved by substituting 0.01 μmole of crystalline egg white lysozyme[2] for the papaya lysozyme preparation and incubating for 30 minutes at 39°. The absorbancy of the unlyzed bacterial suspension (A_0) was determined by omitting the enzyme from the reaction mixture. In both cases 4 M NaOH was added to aliquots of these mixtures as described and the apparent optical density at 440 mμ was measured after 30 minutes.

The lysis of suspensions of *S. lutea* by papaya lysozyme is a complex reaction which does not follow first order kinetics as described for egg white lysozyme (5). Reasonably consistent results are obtained if it is assumed that the reaction is second order with respect to substrate concentration in accord with the usual equation

$$k_2 = \frac{x}{a(a - x)t} \tag{1}$$

where a is the initial substrate concentration, x is the decrement of a occurring in time t, and k_2 is the velocity constant. Although this treatment is not completely satisfactory, evidence to be presented below suggests that its empirical use is justified, at least for the initial phase of the lytic reaction.

From the above, the specific activity (C_2) of papaya lysozyme has been calculated as follows. The initial substrate concentration, $S_0 = A_0 - A_f$. The substrate concentration at time t, $S_t = A_0 - A_t$. Then, if S_0 or a is called 100, $x = S_t/S_0$, and,

[1] We are indebted to Dr. Sherman R. Dickman for the suspensions of *S. lutea* used in this work.

[2] We are grateful to Dr. Edwin E. Hays of the Armour Laboratories for supplying us with a preparation of crystalline egg white lysozyme.

$$k_2 t = \frac{\% \text{ lysis}}{100(100 - \% \text{ lysis})} \quad (2)$$

$$C_2 \equiv \frac{k_2}{\text{mg. protein N per ml. reaction mixture}} \quad (3)$$

The number of lysozyme units is defined as the product of C_2 and the mg. of protein N in the fraction.

Protein concentration was estimated by the turbidimetric method of Bücher (6) with crystalline papaya lysozyme as the standard. For some of the physical studies, the quantity of dissolved protein was measured refractometrically by assuming a refractive index increment of 0.00184 for a 1 per cent solution. These estimates were in good agreement with those performed turbidimetrically or calculated from the protein N value of 17.2 per cent for crystalline lysozyme (see below).

Isolation and Crystallization

The crystalline mercury derivative of papaya lysozyme can be prepared from dried papaya latex as a by-product in the preparation of crystalline papain (4). The lysozyme activity follows the proteolytic activity closely during this preparation and is not separated from it until the papain crystallizes. The mother liquor from this crystallization, Fraction 5a (4), contains papaya lysozyme, which is virtually pure on the basis of specific activity. The same crystalline lysozyme can also be obtained from the mother liquor, Fraction 3a (4), after precipitation of papain with 0.4 saturated ammonium sulfate. Isolation of the lysozyme from Fraction 3a and Fraction 5a is described.[3]

Procedure I (from Fraction 5a)—When 180 gm. of dried papaya latex[4] are used as the starting material, the volume of Fraction 5a is about 425 ml. Enough solid $HgCl_2$ is dissolved in this solution at 4° to bring the mercury concentration to 0.01 M. A voluminous white precipitate (Fraction 5a-1a) appears and is permitted to stand overnight in the cold; this is removed by centrifugation, leaving a clear supernatant solution (Fraction 5a-1).

Fraction 5a-1 is brought to 0.4 saturation with solid ammonium sulfate (250 gm. per liter) and again allowed to stand overnight at 4°. The white precipitate (Fraction 5a-2) formed by this treatment is removed by centrifugation and the supernatant solution (Fraction 5a-2a) is discarded.

[3] The numbers of the fractions given in the text and in Table I are the same as previously given for the isolation of crystalline papain.

[4] We are indebted to the American Ferment Company and to the Wallerstein Laboratories for preparations of commercial papain.

Fraction 5a-2 is resuspended in water at a protein concentration of about 1 per cent and the small amount of insoluble material is removed by gravity filtration on fluted paper. Solid ammonium sulfate is added to the resulting solution until a faint persistent turbidity develops. This is followed by addition of solid $HgCl_2$ (2.7 gm. per liter), and the suspension is stirred slowly in the cold.[5] Crystallization occurs in 2 to 3 days, as indicated by the marked sheen exhibited by the suspension. These crystals appear as hexagonal plates microscopically (Fig. 1).

Procedure II (*from Fraction 3a*)—Solid ammonium sulfate is added to Fraction 3a until 0.8 saturation is reached, when heavy precipitation occurs. This precipitate is removed by gravity filtration on fluted paper and further concentrated by pressing between sheets of Whatman No. 50 filter paper backed by layers of absorbent paper towels. This procedure requires

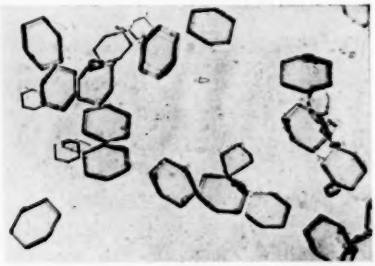


FIG. 1. A preparation of the mercury derivative of crystalline lysozyme as seen under 440 × magnification.

about 24 hours and is performed in the cold. The resulting product is a thick cream-colored paste. This paste is stuffed into dialysis tubing (no more than two-thirds full), and is dialyzed against changes of distilled water until the dialysate is free of sulfate. The resulting solution (Fraction 3a-1) should contain 7 to 9 per cent protein.

Mercuric chloride (2.7 gm. per liter) is added to Fraction 3a-1 and the resulting precipitate (Fraction 3a-1a) removed on fluted paper. The filtrate (Fraction 3a-2) is brought to 0.4 saturation with solid ammonium sulfate (250 gm. per liter) and adjusted to pH 5.5 by addition of 1 M NaOH. After standing overnight at 4°, the precipitate (Fraction 3a-3) is removed by centrifugation and the supernatant solution (Fraction 3a-3a) discarded.

Fraction 3a-3 is redissolved in the minimal amount of water and is treated with $HgCl_2$ and ammonium sulfate, as described for Fraction 3a-1. Crystallization will take place in the resulting suspension after stirring in the cold for 3 to 4 days.

[5] This is conveniently done with a magnetic stirrer.

Recrystallization—Papaya lysozyme may be recrystallized repeatedly by dissolving it in water and adding solid ammonium sulfate (250 gm. per liter) followed by $HgCl_2$ (2.7 gm. per liter). The resulting suspension, if stirred gently at 4°, will exhibit a crystalline sheen in 24 to 28 hours, but stirring has generally been continued for 3 to 4 days to insure completeness of crystallization.

Yield—Table I summarizes the results for a typical isolation of lysozyme obtained from both Fraction 5a (Procedure I) and Fraction 3a (Procedure II) with an initial lot of 180 gm. of dried papaya latex (commercial papain). It should be noted that the yields of crystalline product are modest, being only 10 units from Fraction 5a and 1 unit from Fraction 3a, or about 5 per cent of the original lysozyme activity.[6] However, no attempt was made to develop a procedure which would sacrifice the yield of crystalline papain. The lysozyme may be obtained by the above procedures essentially as a by-product of the papain isolation.

It is noteworthy that the best activity obtained, $C_2 = 0.10$, is only about 3 times the specific lysozyme activity of the initial extract of the latex; for Fraction I, $C_2 = 0.032$.

Inasmuch as the crystalline lysozyme appears to be essentially homogeneous, a C_2 of 0.10 may be tentatively accepted as the activity of the enzyme. On this basis it appears that almost one-third of the soluble protein of the papaya latex is lysozyme. Since papain represents about 7 per cent of papaya extract (4), the two enzymes account for almost 40 per cent of the soluble protein of the commercial dried latex.

Enzymatic Properties

The crystalline lysozyme contains mercury (see below) and does not manifest its full lytic activity unless the mercury is removed. As shown in Table II, the full activation of the mercury derivative is achieved by addition of 0.004 M cysteine. Addition of Versene (ethylenediaminetetraacetate) has no further effect on the activity. The mercury is readily removed by dialysis of the crystalline enzyme against several changes of 0.01 M cysteine, followed by dialysis against water. Table II shows that such a "metal-free" lysozyme is fully active and that further addition of cysteine

[6] It is important to note that assays of lysozyme by the use of the second order velocity constant are rather unsatisfactory with crude preparations of the enzyme. The lysozyme content was frequently higher, when judged by activity measurements, than was to be expected from electrophoretic analysis. For example, Fraction 5 (Table I) is crystalline papain of greater than 95 per cent purity, yet the lytic activity suggests that 35 per cent of the preparation is lysozyme. In view of the crude nature of the lysozyme assay with an organism as the substrate, it is possible that active proteinases such as papain enhance the lytic activity.

TABLE I

Isolation of Crystalline Mercury Derivative of Papaya Lysozyme

A representative run giving yields and activities obtained from 180 gm. of dried latex. Proteolytic activity, C_1, was determined at 39° with α-benzoyl-L-arginin-amide as the substrate in 0.02 M acetate at pH 5.1 containing 0.005 M cysteine and 0.001 M Versene (4). Lysozyme activity, C_2, was measured at 39° in 0.1 M acetate at pH 4.6 with 0.005 M cysteine as the activator and a suspension of *S. lutea* as the substrate. Total activity was calculated by multiplying the specific activity, C_1 or C_2, by the amount of protein N present in the fraction.

Fraction No.	Volume	Total protein	Proteolytic, C_1	Total proteolytic activity	Lysozyme, C_2	Total lysozyme activity
	ml.	*gm.*		*units*		*units*
1	725	41	0.21*	1400	0.032	214
2	820	40	0.23	1510	0.035	230
3		26	0.29	1260	0.045	195
3a	775	17	0.23	625	0.012	32
4		7	0.50	550	0.087	95
4a	755	22	0.19	660	0.030	104
5		1.8	1.33	382	0.037	11
5a	448	4.8	0.32	252	0.100	79
5a-1	575	2.7			0.070	31
5a-1a	Insoluble					
5a-2		0.87			0.080	11
5a-2a	525	2.0			0.019	4
5a-3	Crystals				0.063	
5a-4, 5a-5	Recrystallizations	0.90	None	None	0.07–0.10	10
3a-1	185	17			0.017	47
3a-1a	182	16				
3a-2	192	15				
3a-3	43	0.50			0.019	1.5
3a-3a	43	0.07			0.009	
3a-4	43	0.47			0.026	1.6
3a-5	Crystals	0.28			0.061	2.7
3a-6, 3a-7	Recrystallizations				0.060	
3a-8	Crystals (3×)	0.067	None	None	0.090	1.0

* It may be noted that some of the fractions yielded different values for C_1 from those reported earlier (4). Different batches of dried latex exhibit some variation in yields and specific activities of the fractions. For recrystallized mercuripapain, C_1 values as high as 1.7 have been obtained.

has no effect. Such preparations can be lyophilized with no detectable loss of activity.

The data in Table III show that lysozyme is not inhibited by iodoacetate or by *p*-chloromercuribenzoate; these findings indicate that the enzyme

contains no essential sulfhydryl group. It has also been noted that egg white lysozyme contains no sulfhydryl groups (7). Since Versene and

TABLE II

Activation of Mercury Derivative of Lysozyme by Cysteine

The reactions were performed at 39° in 0.1 M acetate at pH 4.0 with a suspension of *S. lutea* as the substrate. Reactions with the lyophilized "metal-free" lysozyme were run in 0.025 M citrate at pH 4.65.

Preparation	Additions	Protein N	Lysis, 15 min.
		mg. per ml.	*per cent*
Hg derivative	None	0.053	56
" "	5×10^{-5} M cysteine	0.053	53
" "	5×10^{-4} " "	0.053	78
" "	5×10^{-3} " "	0.053	81
Lysozyme ("metal-free")	None	0.004	34
" "	5×10^{-3} M cysteine	0.004	32
" "	5×10^{-3} " " + 1×10^{-3} " Versene	0.004	34

TABLE III

Effect of Metal Ions and Inhibitors on Activity of Lysozyme

The enzyme was a "metal-free" preparation made by dialyzing the mercury derivative against 0.01 M cysteine for 24 hours, followed by dialysis against several changes of distilled water for 48 hours. The reactions were run at 39° in 0.1 M acetate at pH 4.65. A suspension of *S. lutea* was the substrate.

Addition		Lysis, 30 min.
	M	*per cent*
None		44
p-Chloromercuribenzoate	0.0001	46
Iodoacetamide	0.0001	43
NaF	0.01	45
$HgCl_2$	0.0001	24
"	0.001	19
$CoCl_2$	0.001	16
$AgNO_3$	0.001	33
$ZnCl_2$	0.001	50

cysteine do not inhibit the enzyme, it would seem that the enzyme contains no essential metal ion. The lytic activity is inhibited by Hg^{++}, Co^{++}, and Ag^{+} ions. It is striking that, although the enzyme crystallizes with a stoichiometric amount of Hg^{++} (see below), even 0.001 M Hg^{++} produces only a partial inhibition (Table III) and the crystalline mercury derivative manifests a substantial activity without removal of the metal ion. This

suggests that Hg^{++} is only loosely bound to the protein and that the complex dissociates readily in aqueous solution.

It has already been mentioned that a satisfactory assay may be obtained on the basis of second order kinetics for the lytic activity. For a system described by Equation 2, a plot of (100/(100 − per cent lysis)) *versus* time should yield a straight line. Fig. 2 shows that this relationship obtains over at least the first 50 per cent of lysis (the ordinate = 2.0).

The second order rate constant, k_2, is essentially a linear function of protein concentration over a satisfactory range, as indicated by the data in Fig. 3. All of the assays reported were obtained in the region where the linear relationship applies.

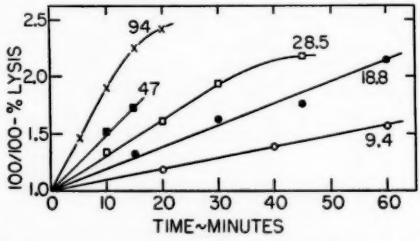


FIG. 2. Effect of lysozyme concentration on the rate of lysis of a suspension of *S. lutea*. The experiments were performed at 39° in 0.1 M acetate buffer at pH 4.65 in the presence of 0.005 M cysteine. The numbers adjacent to each line give the enzyme concentration in mg. of protein N × 10^3 per ml. The linearity of the initial slopes is consistent with a second order reaction.

Fig. 4 shows the activity of lysozyme as a function of pH. The enzyme shows a sharp optimum at pH 4.65. Meyer and coworkers (3) have already noted that *Ficus* lysozyme shows an acidic optimum. In contrast to the plant lysozymes, egg white lysozyme manifests its optimal activity near pH 6 (5). The data in Fig. 4 also demonstrate that there are no specific ion effects such as those observed by Dickman and Proctor (5) for egg white lysozyme.

Comparison of the activity of egg white lysozyme with papaya lysozyme indicates that, under the conditions of our assay at the optimal pH (4.65), 0.023 mg. of protein of papaya lysozyme produces 27 per cent lysis in 10 minutes. Under the same conditions, but at pH 6, it requires only 0.0028 mg. of protein of egg white lysozyme to achieve the same degree of lysis. Thus, the animal lysozyme is 8.2 times more active. Since the two proteins differ in molecular weight (see below), the relative activities may also be compared on a molar basis. Such a computation shows that the egg white lysozyme is 4.8 times more active.

The twice recrystallized papaya lysozyme is devoid of papain activity, as indicated by tests with benzoyl-L-argininamide as substrate (Table I). The procedure was that already described (4). A spectrophotometric assay by the method of Kunitz (8) showed that the lysozyme preparation has no proteolytic action on horse hemoglobin.

It is interesting to note that twice crystallized papain contains some lysozyme activity. However, crystalline mercuripapain (4) has no detectable lytic activity when tested in the presence of cysteine and Versene.

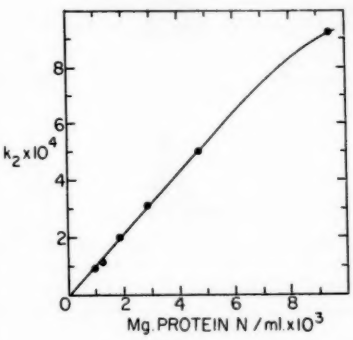


FIG. 3

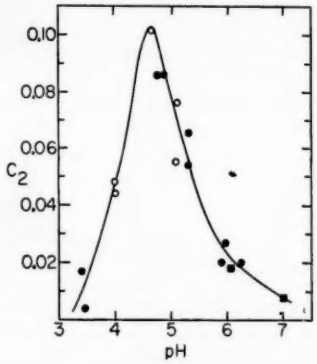


FIG. 4

FIG. 3. Effect of lysozyme concentration on the second order rate constant for lysis of *S. lutea*. The values are taken in part from the experiments shown in Fig. 2.

FIG. 4. Effect of pH on the activity of papaya lysozyme expressed as the second order coefficient, C_2. The buffers employed were 0.1 M acetate (○), 0.025 M citrate (●), and 0.1 M phosphate (■).

Electrophoretic Studies

These observations were made in a Tiselius apparatus equipped with a schlieren scanning device. The runs were made at 1.5° in univalent buffers at an ionic strength of 0.12. The pH values were measured at room temperature with a Cambridge Instrument Company glass electrode standardized with a buffer at pH 4.00 of 0.05 M potassium acid phthalate. Only descending boundaries were used for the measurement of mobility.

Preparations of 2 or 3 times crystallized mercurilysozyme were studied over the range, pH 3.9 to 10.9. The buffer solutions contained 0.02 M cysteine and 0.001 M Versene. The enzyme solutions at pH 10.55 and 10.9 were adjusted to the correct pH with 0.1 M NaOH; the ionic strength was contributed by NaCl. Fig. 5 presents the electrophoretic patterns obtained in representative runs at four pH values. It is evident from the schlieren curves that the lysozyme preparations are essentially monodis-

perse, although occasionally a small amount of an impurity is detectable at levels representing 2 per cent or less of the total area.

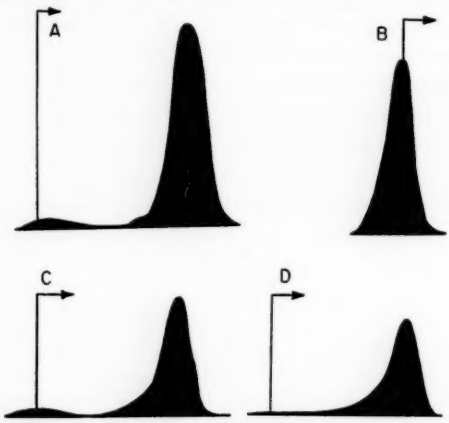


FIG. 5. Electrophoretic diagrams of crystalline papaya lysozyme at various pH values. These are descending patterns obtained at 1.5°. *A*, in acetate buffer at pH 3.9 at a concentration of 0.9 per cent after 166 minutes; *B*, at pH 10.55 in NaCl at 0.6 per cent after 225 minutes; *C*, in acetate at pH 5.8 at 0.3 per cent after 150 minutes; *D*, in Veronal at pH 7.65 at 0.4 per cent after 210 minutes.

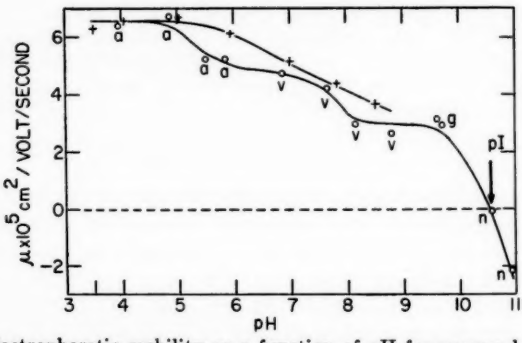


FIG. 6. Electrophoretic mobility as a function of pH for papaya lysozyme (O) and egg white lysozyme (+). The isoelectric point (pI) of papaya lysozyme is shown. The buffers are identified as follows: *a*, acetate; *v*, Veronal; *g*, glycine; and *n*, NaCl.

Fig. 6 shows the mobility of lysozyme as a function of pH. Some data obtained in this laboratory with egg white lysozyme under identical conditions are given for comparison. It is evident that the two proteins have the same high mobility near pH 4 but that the curves are different.

Tanford and Wagner (9) have recently published titration curves for egg white lysozyme. Inspection of these curves shows that they differ greatly from the mobility-pH function for this protein in Fig. 6 and those described earlier (10, 11). It is very likely that these differences indicate a high degree of specific binding of ions by lysozyme. This may also be the cause of the pronounced changes in slope shown by the mobility curve of papaya lysozyme. Papaya lysozyme has an apparent isoelectric point at pH 10.53, whereas egg white lysozyme is isoelectric near pH 11.0 (9).

Sedimentation Studies

A Spinco model E electrically driven ultracentrifuge was used for these studies. The runs were made at 59,780 r.p.m., and at room temperature. The controls and procedures were essentially the same as those in earlier

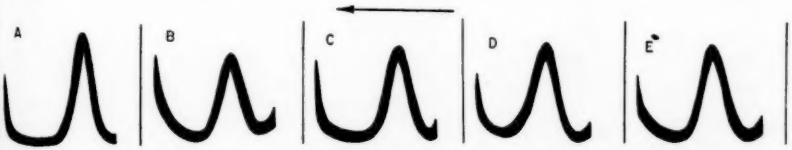


FIG. 7. Sedimentation diagrams obtained with papaya lysozyme at 59,780 r.p.m. *A*, at pH 3.9 in acetate buffer containing cysteine; *B*, at pH 7.9 in Tris-NaCl containing cysteine; *C*, at pH 7.9 with 0.0001 M $HgCl_2$; *D*, at pH 7.9 with the mercury derivative as obtained after recrystallization; *E*, at pH 7.9 with excess $HgCl_2$. The individual pictures are representative of all runs in that only one sedimenting boundary is observed. The sedimentation diagrams illustrated are at the highest protein concentrations shown for the five conditions given in Fig. 8; the capital letters in the two figures correspond.

studies from this laboratory (12–14). The sedimentation data are given in Svedberg units corresponding to water at 20° ($s_{20,w}$), as calculated by the procedure of Svedberg and Pedersen (15).

Preparations of 2 or 3 times recrystallized lysozyme gave sedimentation patterns which appear to be monodisperse. Some typical results obtained under various conditions are shown in Fig. 7.

Data relating sedimentation constant and protein concentration appear in Fig. 8. Curves A and B were obtained in the presence of 0.02 M cysteine and 0.001 M Versene in order to remove bound Hg^{++}; under these conditions, the enzyme manifests its full activity. The experiments in Curve A were performed in acetate buffer of 0.12 ionic strength at pH 3.9, whereas those in Curve B were obtained in Tris-NaCl[7] buffer at pH 7.9 at the same ionic strength. Both sets of data indicate that at infinite dilution $s_{20,w} = 2.57$ S as calculated by the method of least squares.

The measurements in Curve D (Fig. 8) were obtained at pH 7.9 in Tris-

[7] Tris = tris(hydroxymethyl)aminomethane.

NaCl buffer with the crystalline mercury derivative, but no cysteine or Versene was added. It is evident that the data extrapolate to essentially the same value at zero concentration but the slope indicates that, at higher concentrations, some aggregation of the protein occurs.

A preparation of twice crystallized lysozyme was freed of Hg^{++} by the dialysis procedure already given. In tests in Tris-NaCl buffer containing 0.0001 M $HgCl_2$ at pH 7.9, a slight aggregation is produced under these conditions (Fig. 8, Curve C). When a similar experiment was performed with 0.005 M $HgCl_2$, a considerable quantity of the mercury salt crystallized

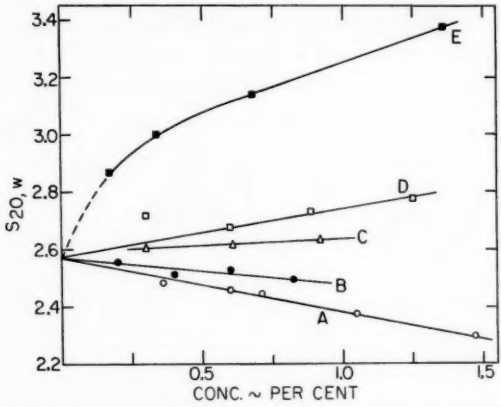


FIG. 8. Sedimentation constant as a function of concentration for papaya lysozyme under various conditions. Curve A, in acetate buffer containing 0.02 M cysteine and 0.001 M Versene at pH 3.9; Curve B, in Tris-NaCl buffer containing the same amounts of Versene and cysteine; Curve C, in Tris-NaCl containing 0.0001 M $HgCl_2$; Curve D is mercurilysozyme in Tris-NaCl at pH 7.9; Curve E is at pH 7.9 in Tris-NaCl with excess $HgCl_2$. The details of the experiments are given in the text.

during the dialysis. However, studies with this preparation, presumably containing a considerable excess of Hg^{++}, showed that extensive aggregation is produced under these conditions (Curve E). Since in the experiments in Curve E as well as those in Curve C the solutions contained a constant amount of Hg^{++}, successive dilutions having been made with the dialysis medium, it is evident that the extent of aggregation is a function of protein concentration as well as of Hg^{++} concentration.

Diffusion Measurements

These studies were performed in the electrophoresis cell by the method of Longsworth (16) from photographs taken by the schlieren scanning method. The results were calculated by the height-area method from the

formula $D = A^2/4\pi H^2 t$, where A is the area under the refractive index curve, H is the maximal height, t the time in seconds, and D the diffusion constant in sq. cm. per sec.

The measurements were made at pH 3.9 in acetate buffer containing 0.02 M cysteine and 0.001 M Versene at a total ionic strength of 0.12. Under these conditions the lysozyme exhibits completely monomeric behavior in the ultracentrifuge (Curve A, Fig. 8). The diffusion studies were made at 1.5° and corrected for the difference in viscosity and temperature in the manner given by Longsworth (16) to give values of $D_{20,w}$. In each of two runs, the results from the two halves of the cell were computed separately, giving duplicate determinations in each case. Six photographs were taken at intervals between 22 and 120 hours after establishing the boundaries. Areas and heights were measured on tracings of projected enlargements of

TABLE IV

Diffusion Constant of Papaya Lysozyme

The measurements were performed at pH 3.9 in acetate buffer of 0.12 ionic strength containing 0.02 M cysteine and 0.001 M Versene.

Run No.	Concentration	$D_{20,w} \times 10^7$
	per cent	*sq. cm. per sec.*
1	0.43	9.29, 9.29
2	1.09	9.14, 9.68
Average		9.35 ± 0.17

the individual diffusion curves. The slopes obtained from plots of $1/H$ against $\sqrt{t}$ gave satisfactory straight lines. The diffusion constants are given in Table IV. The average value for $D_{20,w}$ is $9.35 \pm 0.17 \times 10^{-7}$ sq. cm. per sec.

Molecular Weight

The molecular weight of papaya lysozyme was computed from the sedimentation-diffusion measurements with the aid of the usual formula (15), $M = RTs/D(1 - \bar{V}\rho)$, where T is the absolute temperature, R is the gas constant, ρ is the density of water at 20°, and $\bar{V}$ is the partial specific volume.

$\bar{V}$ is 0.726 as computed from the amino acid composition given below and the specific volumes of the amino acid residues (17). The amide groups were assigned equally as glutaminyl and asparaginyl residues.

With $S = 2.57 \times 10^{-13}$ and $D = 9.35 \times 10^{-7}$, the molecular weight is 24,300. The frictional ratio (f/f_0) computed from these values is 1.19.

Elementary Analysis

Samples of papaya lysozyme which had been recrystallized two or three times were prepared for analysis by washing them three times with 70 per cent ethanol; the final wash fluid gave a negative test for sulfate with $BaCl_2$. The crystals were then washed successively with 95 per cent ethanol, absolute ethanol, acetone (twice), and ether (twice). After each washing, the crystals were centrifuged, the wash fluid was discarded, and the crystals were thoroughly dispersed in the medium. The dried crystals were then allowed to equilibrate in the air at room temperature for 1 week. The moisture content was 6 to 7 per cent in different batches.

The nitrogen content was determined by the Dumas method and the sulfur content by the procedure of Elek and Hill (18); these determinations were performed by Dr. A. Elek of Los Angeles. Duplicate determinations were in close agreement and showed 17.2 per cent nitrogen and 1.88 per cent sulfur.

The analysis indicates a content of 14 atoms of sulfur for 23,900 gm. of protein or 15 atoms for 25,600 gm. However, the studies of amino acid composition given below indicate the presence of 4 moles of methionine and 8 half cystine residues or a total of 12 atoms of sulfur. The presence of more sulfur than expected from the amino acid composition is not surprising in view of the basic character of lysozyme and the method of crystallization of the enzyme from slightly acidic solutions of ammonium sulfate. It is very likely that 2 or 3 moles of inorganic sulfate are present in the crystalline protein.

The mercury content of two samples of lysozyme was determined by the method of Laug and Nelson (19) with dithizone. All the precautions and controls recommended by these investigators were followed. Four separate analyses gave 2.34, 2.36, 2.28, and 2.38 per cent mercury for an average of 2.34 per cent for the anhydrous protein. This indicates that 1 mole of mercury is combined with 8570 gm. of lysozyme. For a content of 3 moles of mercury, the calculated molecular weight is 25,700 in good agreement with the estimate from sedimentation-diffusion data of 24,300.

Amino Acid Composition

The lysozyme used for these studies was a 3 times recrystallized preparation of the mercury derivative which was washed and dried in the manner described for the elementary analyses. The hydrolysates were prepared and worked up as already published for carboxypeptidase (20) and papain (21). The amino acid estimations were performed on Dowex 50-X8 by the chromatographic methods of Moore and Stein (22) with the minor changes already given (20). Typical runs with the elution curves with long and

short columns for a preparation of lysozyme hydrolyzed for 70 hours at 105° are shown in Figs. 9 and 10.[8]

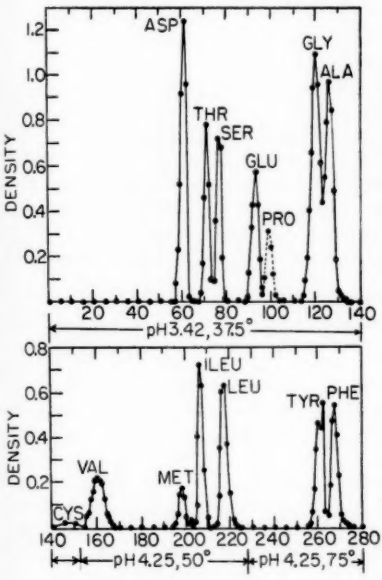


FIG. 9

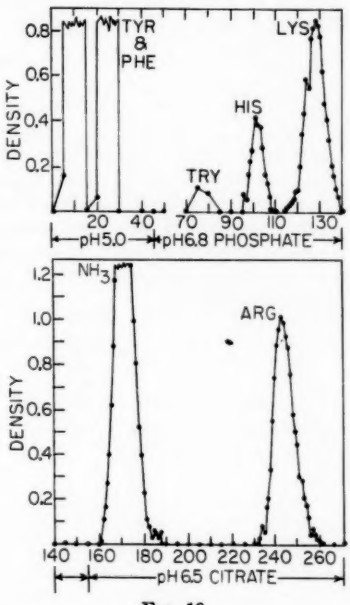


FIG. 10

FIG. 9. Elution curves for a 70 hour hydrolysate of crystalline papaya lysozyme on a 0.9 × 100 cm. column of Dowex 50-X8. The ninhydrin color yields are given for the 1 ml. fractions as optical density and have been corrected for the base-line color but not for the differences in color yield of the various amino acids. The color produced by proline (PRO) was read at 440 mμ; for the other substances at 570 mμ. *ASP* = aspartic acid, *THR* = threonine, *SER* = serine, *GLU* = glutamic acid, *GLY* = glycine, *ALA* = alanine, *CYS* = cystine, *VAL* = valine, *MET* = methionine, *ILEU* = isoleucine, *LEU* = leucine, *TYR* = tyrosine, and *PHE* = phenylalanine.

FIG. 10. Elution curve for a 70 hour hydrolysate of lysozyme for the basic amino acids and ammonia on a 0.9 × 15 cm. column of Dowex 50. Base-line color has been subtracted but no correction has been applied for the color yields of the different substances. *TRY* = tryptophan, *HIS* = histidine, *LYS* = lysine, *ARG* = arginine, *TYR* = tyrosine, and *PHE* = phenylalanine.

The data are given in Table V for analyses after 20, 70, and 140 hours of hydrolysis at 105°. As shown in Fig. 9 and Fig. 10, some of the cystine and tryptophan remain after the prolonged hydrolysis, but no attempt was

[8] We are indebted to Mrs. Vina Buettner-Janusch for these determinations.

made to estimate these. Tryptophan was determined separately by the colorimetric method of Spies (23). Cysteic acid was estimated after performic acid oxidation and hydrolysis as described by Schram, Moore, and Bigwood (24). The separation of cysteic acid was performed on a 0.9

TABLE V

Amino Acid Recoveries from Hydrolysates of Lysozyme

The data are given as gm. of amino acid residue per 100 gm. of anhydrous, ash-free protein. The values for serine, threonine, methionine, and ammonia in the last column were obtained by linear extrapolation to zero time of hydrolysis. Other values omitted from the averages are given in parentheses. Values for the first thirteen amino acids were obtained on a 0.9 × 100 cm. column of Dowex 50, whereas the basic amino acids and ammonia were separated on a 0.9 × 15 cm. column. Cystine was determined separately as cysteic acid (24). Tryptophan was estimated colorimetrically (23).

Amino acid	Time of hydrolysis					Average or extrapolated
	20 hrs.		70 hrs.		140 hrs.	
Aspartic acid	10.35	10.44	10.65	10.26	10.14	10.37 ± 0.14
Threonine	5.04	4.91	4.34	3.97	(4.53)	5.31 ± 0.13
Serine	4.90	5.03	3.29	2.95	(3.70)	5.70 ± 0.12
Glutamic acid	6.31	5.26	6.31	5.72	5.64	5.85 ± 0.37
Proline	7.32	6.98	(5.93)	7.03	7.43	7.19 ± 0.19
Glycine	6.25	5.54	6.37	6.34	6.10	6.12 ± 0.24
Alanine	6.08	6.57	5.82	5.70	6.15	6.06 ± 0.24
Valine	(3.04)	(2.65)	3.09	3.05	3.03	3.06 ± 0.02
Methionine	2.04	(1.16)	1.97	1.65	1.65	2.10 ± 0.08
Isoleucine	(5.01)	(4.41)	5.24	5.36	5.09	5.23 ± 0.09
Leucine	(6.34)	5.42	5.55	5.63	5.45	5.51 ± 0.08
Tyrosine	8.53	8.86	7.93	9.07	8.09	8.50 ± 0.39
Phenylalanine	6.47	7.20	6.96	7.30	6.99	6.98 ± 0.22
Histidine	1.70	1.50	1.46			1.55 ± 0.10
Lysine	5.03	5.46	5.19			5.23 ± 0.16
Ammonia	1.49	1.61	2.10			1.33 ± 0.06
Arginine	8.88	8.22	8.24			8.45 ± 0.29
Half cystine	3.01	3.04				3.03 ± 0.02
Tryptophan						5.11

× 15 cm. column of Dowex 50-X8; this amino acid emerges at the column volume in two or three 1 ml. fractions when a buffer solution at pH 3.4 is used. The values given in Table V are actual recoveries of cysteic acid as determined by the colorimetric ninhydrin method (22).

The data in Table V show, in general, that the recoveries of serine, threonine, and methionine decrease with prolonged hydrolysis, although the values for serine and threonine obtained after 140 hours are higher than

expected. An estimate of the initial content of these three amino acids in the protein was obtained by linear extrapolation to zero time of hydrolysis as described earlier (20, 21). Values omitted from these calculations are given in parentheses in Table V.

It may be noted that the amount of serine and threonine destroyed during hydrolysis for 70 hours, as calculated from the linear extrapolation, is 4.2×10^{-4} mole per 100 gm. of protein. The excess ammonia produced, computed in the same manner, is 4.8×10^{-4} mole. The accord between these values suggests that the amounts of these amino acid residues and the amide content of the protein as calculated from the extrapolation are reasonable estimates. Similar results have already been described for other proteins analyzed in this laboratory (20, 21).

The recoveries of valine and isoleucine obtained at 20 hours are low and have been omitted from the averages. It is to be expected that peptide bonds involving the carboxyl groups of these amino acids are slowly hydrolyzed (20, 21, 25, 26).

In general, the determinations are in satisfactory agreement and, for the values used, the average deviations are less than 5 per cent, with the exception of those for glutamic acid and histidine, which are slightly higher.

Composition of Papaya Lysozyme

Estimates of the composition of papaya lysozyme are given in Table VI. The average molecular weight has been computed on the basis of the methionine content, which indicates approximately 4 residues of this amino acid in 1 mole of protein of 25,000 molecular weight. The average molecular weight computed for all the residues (except cystine and ammonia) is 24,745, in excellent agreement with the value estimated from sedimentation-diffusion measurements, 24,300, and from a content of 3 moles of mercury, 25,700.

In terms of the measured nitrogen content of 17.2 per cent, the recovery of nitrogen calculated from the amino acid determinations and the residue weight recovery are slightly high, but are in accord with the estimated precision of the determinations. The protein does not appear to contain substances other than the usual amino acids.

In general, the calculated number of those amino acids present in small amount is reasonably satisfactory. It has already been noted that small fluctuations in base-line color cause greater uncertainties in determining the content of these amino acids than those present in larger amounts (20, 21). Nevertheless, there appears to be little doubt concerning the number of residues of methionine, histidine, and tryptophan.

We have given the actual recovery of cystine, estimated as cysteic acid, and this value is undoubtedly low since Schram *et al.* (24) indicate that

their procedure yields a recovery of only about 90 per cent of the theoretical amount of cystine. In our experience, recoveries of 80 to 90 per cent have been obtained with cystine itself. Since sulfhydryl groups appear to be absent from lysozyme, it is likely that 8 half cystine (or 4 cystine)

TABLE VI

Composition and Molecular Weight of Papaya Lysozyme

Amino acid	Amino acid residue per 100 gm. protein	Amino acid per 100 gm. protein	N	N as	Minimal mol. wt.	Calculated mol. wt.	Calculated no. of residues for M = 24,745	Assumed no. of residues
	gm.	*gm.*	*gm.*	*per cent total N*				
Aspartic acid	10.37	11.99	1.26	7.33	1110	24,420	22.3	22
Threonine	5.31	6.26	0.74	4.30	1904	24,752	13.0	13
Serine	5.70	6.88	0.92	5.35	1528	24,448	16.2	16
Glutamic acid	5.85	6.67	0.63	3.66	2207	24,277	11.2	11
Proline	7.19	8.52	1.04	6.05	1350	24,300	18.3	18
Glycine	6.12	8.05	1.50	8.72	933	24,258	26.5	26
Alanine	6.06	7.59	1.19	6.92	1173	24,633	21.1	21
Valine	3.06	3.62	0.43	2.50	3239	25,912	7.6	8
Methionine	2.10	2.39	0.22	1.28	6248	24,992	4.0	4
Isoleucine	5.23	6.06	0.65	3.78	2164	23,804	11.4	11
Leucine	5.51	6.39	0.68	3.95	2054	24,648	12.0	12
Tyrosine	8.50	9.44	0.73	4.24	1920	24,960	12.9	13
Phenylalanine	6.98	7.83	0.66	3.84	2109	25,308	11.7	12
Histidine	1.55	1.66	0.45	2.62	8852	26,556	2.8	3
Lysine	5.23	5.96	1.14	6.63	2451	24,510	10.1	10
Ammonia	1.33*	1.41*	1.16	6.74	1204	25,284	20.6*	21*
Arginine	8.45	9.42	3.03	17.62	1849	24,037	13.4	13
Half cystine	3.03	3.56	0.42	2.44	3373	26,984	7.3	8
Tryptophan	5.11	5.60	0.77	4.48	3644	25,508	6.8	7
Total	101.35	117.89	17.62	102.45		24,745†	228.6	228

* These values are omitted from the totals.

† Average for all the values given except for half cystine and ammonia.

residues are present in this protein. The difference between the measured and calculated sulfur content has been mentioned above.

The high content of basic amino acids and of amide groups is in accord with the basic properties of lysozyme. The sum of arginine, histidine, and lysine is 26 residues. The sum of glutamic and aspartic acids, 33 residues, minus the twenty-one amide groups, yields only twelve free carboxyl groups from these residues. Presumably the thirteen phenolic groups of tyrosine would be in the ionic form at pH 10.5, the isoelectric point of the enzyme.

Amino acid analyses of the lysozymes of egg white (7) and spleen (27) show that these enzymes of animal origin are very similar. The papaya lysozyme resembles these in its relatively low content of histidine and glutamic acid and in its relatively high content of arginine, tryptophan, and aspartic acid; these findings aided in the identification of the plant lysozyme. However, the egg white enzyme is much richer in arginine, tryptophan, aspartic acid, and cystine. The plant enzyme has a much higher content of phenylalanine, tyrosine, and proline.

A comparison of the two crystalline enzymes from papaya latex, papain and lysozyme, whose composition is known reveals that they may be readily distinguished. Papain (21) lacks methionine, contains only 1 resi-

TABLE VII

Recovery of DNP-Amino Acids after Hydrolysis of DNP-Lysozyme

Hydrolysis of the DNP protein was performed at 105° in 6 N HCl. The data are given in moles of DNP derivative per mole of protein. Method I was that of Levy and Chung (30); Method II was that described by Thompson (29).

Time of hydrolysis	DNP-glycine by Method I	DNP-glycine by Method II	ε-DNP-lysine by Method II
hrs.			
4	0.14		
8	0.20		
12	0.26	0.34	8.0
16	0.17	0.25	
20	0.11		7.3

due of histidine, and has much less proline and phenylalanine, to cite only the most noteworthy differences.

Free Amino Groups

Some preliminary studies of the free amino groups of papaya lysozyme were performed by the methods of Sanger (28). The general procedures followed for the preparation of the dinitrophenylprotein (DNP protein), hydrolysis, and the separation and identification of the DNP-amino acids were those described earlier (29).

The only DNP-amino acids which could be identified by separation on columns (29) or by paper chromatography (30) were DNP-glycine and ε-DNP-lysine, and trace amounts of DNP-aspartic acid. Table VII shows the recoveries of DNP-glycine obtained by the method of Levy and Chung (30) after different times of hydrolysis. Separate experiments in which DNP-glycine and ε-DNP-lysine were separated by chromatography on Celite columns (29) and estimated spectrophotometrically are also given for two different times of hydrolysis. It is apparent from the results that low

recoveries of ε-DNP-lysine were obtained in comparison with the estimate of 10 lysine residues found by resolution of hydrolysates on Dowex 50. It is well known that destruction of DNP derivatives is greatly increased in proteins which are rich in tryptophan (31), as is the case for papaya lysozyme.

The recovery of DNP-glycine poses an additional problem inasmuch as this DNP derivative is more labile than most DNP-amino acids to acid hydrolysis. Sanger (28) has found that hydrolysis of DNP-glycine in the presence of insulin, which does not contain tryptophan, results in only a 25 per cent recovery after 12 hours hydrolysis in 6 N HCl. On this basis, our results would indicate that papaya lysozyme contains one *N*-terminal glycine per mole of protein. There is reason to believe, however, that the destruction factors in our experiments were larger because of the lower than expected recovery of ε-DNP-lysine, approximately 80 per cent rather than the 95 per cent expected from the studies of Porter and Sanger (32). Furthermore, an experiment in which DNP-glycine was added to DNP-lysozyme, and hydrolysis was performed for 12 hours in 6 N HCl, gave a recovery of only 22 per cent of the theoretical amount of added glycine. When this correction factor is applied, a yield of 1.55 moles of *N*-terminal glycine is obtained.

From these data it appears that papaya lysozyme may consist of two peptide chains each with *N*-terminal glycine; however, the extensive destruction of DNP-glycine necessitates some caution in accepting this value. Other methods will have to be studied in order to determine conclusively the number of *N*-terminal glycine residues. In any case, it is striking that the plant enzyme is different from the animal lysozymes which possess a single *N*-terminal lysine per mole of protein (31, 33).

DISCUSSION

In his description of lysozyme and his study of its biological distribution, Fleming (1, 2) emphasized the important rôle which this enzyme probably plays in the defense of the animal organism against many air-borne bacteria. He also noted that many plants contain lysozyme, but that the activities are much weaker than in the rich animal sources. The existence of rich plant sources of this enzyme suggests a similar important rôle for lysozyme in the defense of plants. Presumably in species like papaya and *Ficus*, which grow in a tropical environment, the lysozymes aid in preventing invasion of the unripe fruits by saprophytic organisms. Inasmuch as the proteolytic enzymes of these fruits can also attack many organisms, including some living animals, these plants appear to be equipped with a strong defense against parasitic invaders.

It is certainly noteworthy that enzymes with the ability to lyze certain bacteria are present in both animals and plants. The work of Meyer,

Hahnel, and Steinberg (3) indicates that both types of enzymes depolymerize the same mucopolysaccharide and hydrolyze this fraction with the liberation of acetyl hexosamine.

The high content of lysozyme in egg white (7) and in spleen (27) has been noted. On the basis of admittedly not very precise assays of activity, lysozyme appears to comprise about one-third of the soluble protein of papaya latex. The electrophoretic Component 3 given by Kimmel and Smith ((4) Table II) is the fraction in which lysozyme is found; this represents about 65 per cent of the total protein. However, this fraction contains chymopapain and probably other proteins also, so that it is not possible from the electrophoretic analysis alone to estimate the lysozyme content of papaya latex with any certainty. Nevertheless, both types of estimate, lytic activity and electrophoretic analysis, are consistent with a high content of lysozyme in the latex. Meyer *et al.* (3) have estimated that the lysozyme content of crude ficin is about 20 per cent.

The properties of the papaya lysozyme reveal many interesting features, perhaps the most noteworthy being that which led to this study; namely, its ability to form a crystalline mercury derivative, and the fact that, as yet, we have been unable to prepare crystals of this enzyme in any other form. Other crystalline mercury derivatives of proteins have been prepared; enolase (34), mercaptalbumin (35), and papain (4), but in the last two cases there is evidence which suggests that the mercury binds with sulfhydryl, whereas for papaya lysozyme the evidence indicates that such groupings are not involved. This is suggested by the findings that sulfhydryl agents do not inhibit the enzyme, and that the combination of mercury with the protein is much more labile than might be expected for a mercury-sulfhydryl binding, as shown by the fact that the protein-metal complex dissociates on dilution or simple dialysis against water. Rather than a sulfhydryl binding, it is more likely that Hg^{++} is bound to carboxyl or imidazole groups. The analytical findings of 3 moles each of histidine residues, mercury atoms, and sulfur, presumably as inorganic sulfate, are suggestive. A type of weak binding in which each atom of mercury is bound both to imidazole and to inorganic sulfate would be in accord with all of the known properties of these substances.

A comparison of papaya lysozyme with those from egg white and spleen reveals many interesting differences and similarities, some of which have already been mentioned. One further point worth noting is that the animal and plant enzymes have similar, although not identical, high isoelectric points, and that the number of arginine and lysine residues in both types of enzyme is very similar. However, in the animal enzymes arginine predominates (7), whereas papaya lysozyme contains almost equivalent amounts of these two basic amino acids. The similarity in biological function of these bases is strikingly revealed by the studies of du Vigneaud and

his coworkers (36), who have demonstrated that, in the vasopressins of swine and bovine glands, the hormone of the former contains a single lysine residue, whereas that of the latter contains arginine in the same locus in the peptide chain. A similar biological relationship is seen in the specificity of trypsin which has been found to hydrolyze only peptide bonds involving arginine or lysine (37).

It is to be hoped that the fundamental similarities in the properties of the animal and plant lysozymes shown in their basicity and wealth of basic amino acids, as well as of aspartic acid and tryptophan, may eventually aid in understanding the enzymatic properties of these proteins.

SUMMARY

1. Two methods are described for obtaining a crystalline mercury derivative of lysozyme from dried papaya latex. The enzyme appears to represent about one-third of the soluble protein of the latex.

2. The enzyme is not inhibited by sulfhydryl reagents or poisons of metal ions. Hg^{++} is inhibitory and must be removed from the crystalline material for full activity. Lysis of *Sarcina lutea* follows second order kinetics for the initial 50 per cent of lysis. The enzyme is optimally active at pH 4.65. It is about one-fifth as active as egg white lysozyme on a molar basis.

3. The enzyme is essentially monodisperse on electrophoresis over the pH range from 3.9 to 10.9. The mobility curve of the papaya enzyme differs from that of egg white lysozyme. Papaya lysozyme is isoelectric at pH 10.5.

4. Papaya lysozyme has a sedimentation constant ($s_{20,w}$) of 2.57 S. In the presence of Hg^{++} some aggregation occurs. The diffusion constant ($D_{20,w}$) is 9.35×10^{-7} sq. cm. per sec. The partial specific volume ($\bar{V}$) computed from the amino acid composition is 0.726; the molecular weight 24,300.

5. A study of its composition indicates 3 moles of mercury, 3 moles of inorganic sulfate, and 3 moles of histidine, which suggests a relationship among these components. The amino acid composition indicates a basic protein rich in arginine, lysine, aromatic amino acids, and aspartic acid.

6. The protein contains *N*-terminal glycine unlike animal lysozymes which possess *N*-terminal lysine.

7. The possible significance of plant lysozymes is discussed. A comparison of the properties of the animal and plant enzymes is given.

BIBLIOGRAPHY

1. Fleming, A., *Proc. Roy. Soc. London, Series B*, **93**, 306 (1922).
2. Fleming, A., *Proc. Roy. Soc. Med.*, **26**, 71 (1932).
3. Meyer, K., Hahnel, E., and Steinberg, A., *J. Biol. Chem.*, **163**, 733 (1946).

4. Kimmel, J. R., and Smith, E. L., *J. Biol. Chem.*, **207**, 515 (1954).
5. Dickman, S. R., and Proctor, C. M., *Arch. Biochem. and Biophys.*, **40**, 364 (1952).
6. Bücher, T., *Biochim. et biophys. acta*, **1**, 292 (1947).
7. Fevold, H. L., *Advances in Protein Chem.*, **6**, 188 (1951).
8. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947).
9. Tanford, C., and Wagner, M. L., *J. Am. Chem. Soc.*, **76**, 3331 (1954).
10. Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580 (1940).
11. Alderton, G., Ward, W. H., and Fevold, H. L., *J. Biol. Chem.*, **157**, 43 (1945).
12. Smith, E. L., Brown, D. M., Fishman, J. B., and Wilhelmi, A. E., *J. Biol. Chem.*, **177**, 305 (1949).
13. Smith, E. L., Brown, D. M., and Laskowski, M., *J. Biol. Chem.*, **191**, 639 (1951).
14. Smith, E. L., and Brown D. M., *J. Biol. Chem.*, **195**, 525 (1952).
15. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
16. Longsworth, L. G., *Ann. New York Acad. Sc.*, **41**, 267 (1941).
17. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York (1943).
18. Elek, A., and Hill, D. W., *J. Am. Chem. Soc.*, **55**, 3479 (1933).
19. Laug, E. P., and Nelson, K. W., *J. Assn. Official Agr. Chem.*, **25**, 399 (1942).
20. Smith, E. L., and Stockell, A., *J. Biol. Chem.*, **207**, 501 (1954).
21. Smith, E. L., Stockell, A., and Kimmel, J. R., *J. Biol. Chem.*, **207**, 551 (1954).
22. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948); **192**, 663 (1951).
23. Spies, J. R., *Anal. Chem.*, **20**, 30 (1948); **22**, 1447 (1950).
24. Schram, E., Moore, S., and Bigwood, E. J., *Biochem. J.*, **57**, 33 (1954).
25. Harfenist, E. J., and Craig, L. C., *J. Am. Chem. Soc.*, **74**, 4216 (1952).
26. Harfenist, E. J., *J. Am. Chem. Soc.*, **75**, 5528 (1953).
27. Jollès, G., and Fromageot, C., *Biochim. et biophys. acta*, **11**, 95 (1953); **14**, 219 (1954).
28. Sanger, F., *Biochem. J.*, **39**, 507 (1945).
29. Thompson, E. O. P., *J. Biol. Chem.*, **207**, 563 (1954).
30. Levy, A. L., and Chung, D., *Nature*, **174**, 126 (1954).
31. Thompson, A. R., *Nature*, **168**, 390 (1951).
32. Porter, R. R., and Sanger, F., *Biochem. J.*, **42**, 287 (1948).
33. Jollès, P., and Fromageot, C., *Biochim. et biophys. acta*, **14**, 228 (1954).
34. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941).
35. Hughes, W. L., Jr., *J. Am. Chem. Soc.*, **69**, 1836 (1947).
36. du Vigneaud, V., Lawler, H. C., and Popenoe, E. A., *J. Am. Chem. Soc.*, **75**, 4880 (1953).
37. Bergmann, M., and Fruton, J. S., *Advances in Enzymol.*, **1**, 63 (1941).

ASPARTATE METABOLISM AND ASPARAGINE SYNTHESIS IN PLANT SYSTEMS*

By GEORGE C. WEBSTER and J. E. VARNER†

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California)

(Received for publication, December 6, 1954)

The synthesis of glutamine according to the reaction

$$\text{Glutamate} + \text{ATP} + \text{NH}_3 \rightleftharpoons \text{glutamine} + \text{ADP} + \text{P}_i$$

has been well established in plant (1, 2), animal (3, 4), and bacterial (5, 6) systems.[1] However, as several investigators have reported (3, 4, 6), when aspartate has been substituted for glutamate in the above reaction, no evidence for asparagine synthesis could be obtained. Particularly noteworthy is the report of Elliott (4) that extracts of lupine seedlings, which form considerable glutamyl hydroxamate in the presence of glutamate, NH_2OH, ATP, and magnesium ions, fail to form any aspartyl hydroxamate on substitution of aspartate for glutamate. In contrast to this, Black and Gray (7) have demonstrated that the formation of aspartyl hydroxamate is catalyzed by a yeast enzyme which normally forms aspartyl phosphate (but apparently not asparagine) by the following reaction

$$\text{Aspartate} + \text{ATP} \longrightarrow \text{aspartyl phosphate} + \text{ADP}$$

Despite the considerable interest in the manner of asparagine synthesis, there has been but little information on the nature of the reaction.

Etiolated lupine seedlings have been reported (8) to accumulate as much as 25 per cent of their dry weight in the form of asparagine, and, as the work of both Vickery and Pucher (9) and Meiss (10) has demonstrated, provide excellent experimental material for the study of asparagine synthesis. The present paper reports studies with both intact lupine seedlings and cell-free extracts. The results indicate that asparagine can be formed in these systems by a reaction analogous to that involved in glutamine formation.

* Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company, Inc., and by a grant-in-aid to one of us (J. E. V.) from the Charles F. Kettering Foundation. Presented before the annual meeting of the American Society of Plant Physiologists, Gainesville, Florida, 1954.

† Present address, Department of Agricultural Biochemistry, The Ohio State University, Columbus, Ohio.

[1] The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; Tris, tris(hydroxymethyl)aminomethane.

XUM

EXPERIMENTAL

Lupine seedlings were grown and homogenates prepared from these and other tissues as described previously (11). Extracts of wheat germ and of wheat germ acetone powder were also prepared as described earlier (12).

Ethanol precipitation of the enzyme protein was carried out by mixing 3 volumes of ethanol with the cell-free extract at 1°, removing the protein by centrifugation, and dissolving it in 0.5 volume of 0.1 M Tris-HCl (pH 7.5). Ethanol was removed from the protein-free supernatant fluid with a low temperature rotating evaporator.

The composition of the various reaction systems is described with Tables I to III and Figs. 1 to 6. The formation of hydroxamates of aspartate and other amino acids was determined by the method of Lipmann and Tuttle (13). The formation of radioactive aspartate and radioactive asparagine was measured by their chromatographic isolation in the following manner. The reaction systems (deproteinized with 6 per cent trichloroacetic acid and containing 5 mg. each of carrier aspartate and asparagine) were subjected to a preliminary separation on a 1 × 30 cm. Dowex 50 column. With 1.5 N HCl as the eluent, aspartate and asparagine migrate as a single peak in the position normally observed for aspartate under these conditions. This treatment serves to separate aspartate and asparagine from organic acids formed by deamination of aspartate and from β-alanine formed by aspartate decarboxylation. The combined fractions containing aspartate and asparagine were evaporated to dryness under vacuum in a low temperature, rotating evaporator and taken up in a small volume of 0.2 M ammonium acetate of pH 5.0. Aspartate and asparagine were separated from each other on a 1 × 30 cm. column of Amberlite IR-4B with 0.2 M ammonium acetate (pH 5.0) as the eluent (14). A typical separation is presented in Fig. 1. The radioactivities of the asparagine and aspartate fractions were determined by standard techniques with an end window Geiger-Müller tube and scaling circuit.

Preparation of β-Alanylamide—β-Bromopropionyl chloride was added slowly with constant stirring to a 10-fold excess of concentrated ammonium hydroxide. The solution was allowed to stand overnight at room temperature, concentrated to a thick syrup by reduced pressure distillation, and precipitated with ethanol. The product was then recrystallized three times from ethanol.

Results

Early experiments confirmed the reports of previous investigators (3, 4, 6) that aspartyl hydroxamate is not formed by tissue extracts under the conditions normally optimal for glutamyl hydroxamate formation. If,

however, the concentrations of both aspartate and hydroxylamine are increased about 10-fold, it then becomes possible to demonstrate the formation of aspartyl hydroxamate. Table I illustrates that a variety of plant tissue extracts are capable of catalyzing the formation not only of aspartyl but also of β-alanyl hydroxamate. It should be noted that all of the components of the reaction system, amino acid, hydroxylamine, ATP, magnesium ions, and tissue extract, are necessary for optimal hydroxamate formation. The ability of the various extracts to bring about hydroxamate formation varies widely, the highest activity being found in wheat germ extracts and the lowest (no detectable activity) in spinach leaf extracts.

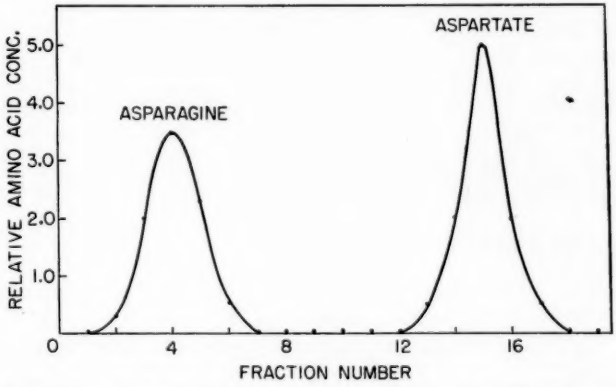


FIG. 1. Separation of aspartate and asparagine on an Amberlite IR-4B column. 2 ml. fractions were collected from a 1 × 30 cm. column with 0.2 M ammonium acetate of pH 5.0 as the eluting agent.

The fact that β-alanine is activated suggests that asparagine formation might proceed by the initial formation of β-alanylamide, followed by the carboxylation of this amide to form asparagine. The data of Table II indicate, however, that this is not the case. The carboxylative formation of asparagine from β-alanylamide is considerably less than the formation of asparagine from free β-alanine, indicating that the amide is probably hydrolyzed to free β-alanine before conversion to asparagine. The direct conversion of β-alanylamide to asparagine, therefore, seems unlikely. The results of the carboxylation experiments instead suggest that aspartate itself is a precursor of asparagine. This suggestion is supported by the data of Fig. 2, in which intact lupine seedlings were incubated with ammonium chloride and aspartate-C^{14} for 3 hours. The major fraction of radioactivity in the cells was found in asparagine and considerably less in other fractions (including aspartate itself).

The transformation of aspartate-C^{14} to asparagine-C^{14} can be demonstrated with cell-free extracts of either lupine seedlings or wheat germ. As is illustrated in Table III, wheat germ extracts readily carry out asparagine synthesis in the presence of aspartate, ATP, and ammonium and magnesium ions. Almost identical results have been obtained with lupine seedling

TABLE I

*Formation of Aspartyl and β-Alanyl Hydroxamate by Extracts of Various Plant Tissues**

Tissue	Aspartyl hydroxamate formed†	β-Alanyl hydroxamate formed†
Lupine seedlings	0.18	0.19
Germinating peas	0.08	0.08
Wheat germ‡	0.25	0.25
Spinach leaves‡	0.00	0.00
Yeast‡	0.16	0.16

* Assay system, 0.05 M Tris-HCl (pH 8.3), 0.10 M amino acid, 0.40 M hydroxylamine, 0.01 M $MgSO_4$, 0.005 M ATP, and 0.5 ml. of tissue extract in a total volume of 4 ml.

† Micromoles per hour per mg. of protein at 38°.

‡ Acetone powder.

TABLE II

Carboxylation Reactions Involving β-Alanine and β-Alanylamide

System*	Aspartate	Asparagine
	c.p.m.	*c.p.m.*
β-Alanine + $C^{14}O_2$	45,380	3063
β-Alanylamide + $C^{14}O_2$	5,160	951

* The complete system contained 0.05 M Tris-HCl (pH 7.5), 0.02 M amino acid or amide, 0.01 M $NaHC^{14}O_3$, 0.01 M $MgSO_4$, 0.01 M NH_4Cl, 0.005 M ATP, 0.01 mg. each of biotin, thiamine pyrophosphate, and pyridoxal phosphate, and 0.5 ml. of wheat germ extract in a total volume of 2 ml.

extracts. Even in these undialyzed preparations, requirements for ATP, NH_4Cl, and $MgSO_4$ are apparent. The strong inhibition of synthesis caused by cyanide and dinitrophenol suggests the participation of oxidative phosphorylation in the synthetic process, presumably in the regeneration of ATP. The strong inhibition caused by *p*-chloromercuribenzoate indicates a participation of sulfhydryl groups in the synthetic reaction. It appears, therefore, that asparagine can be formed by these cell-free extracts by a process similar to that involved in glutamine synthesis.

The ethanol-precipitated protein forms asparagine less actively (in the

presence of aspartate, ATP, NH_4Cl, and $MgSO_4$) than the crude enzyme extract (Table III). When the protein-free supernatant fluid (after re-

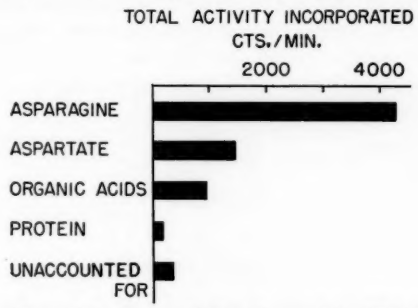


FIG. 2. Incorporation of the radioactivity of aspartate-C^{14} into various fractions of 5 day-old etiolated lupine seedlings incubated for 3 hours at 38° with 0.05 M aspartate-C^{14} (uniformly labeled), containing a total activity of 850,000 c.p.m., and 0.03 M NH_4Cl. Aspartate and asparagine were isolated as described earlier. Protein was isolated by precipitation with trichloroacetic acid and treated as described elsewhere (12). The fraction designated as organic acids contains the total ether-soluble fraction of the cellular material.

TABLE III

Characteristics of Asparagine-Forming System of Wheat Germ Extracts

System*	Asparagine-C^{14} formed
	μmole
Complete	0.60
No NH_4Cl	0.18
" ATP	0.16
" $MgSO_4$	0.45
" supernatant fluid	0.20
" enzyme	0.01
+ HCN	0.35
+ dinitrophenol	0.24
+ *p*-chloromercuribenzoate	0.09

* The complete system contained 0.08 M Tris-HCl (pH 7.5), 0.05 M aspartate-C^{14}, 0.04 M NH_4Cl, 0.002 M ATP, 0.005 M $MgSO_4$, 0.1 ml. of ethanol-precipitated enzyme from wheat germ extract, and 0.4 ml. of protein-free supernatant solution from enzyme purification, in a total volume of 1 ml. The inhibitor concentration in each case was 0.001 M. Temperature, 38°; time, 3 hours. Asparagine formation was determined by radioactive assay after separation of aspartate and asparagine chromatographically.

moval of ethanol) is recombined with the protein, more than 90 per cent of the original activity is regained. It is evident, therefore, that some additional non-protein factor present in the extract is required for optimal

activity. It is not clear as yet whether this factor participates directly in the synthetic reaction or whether it is merely a glycolytic intermediate that participates in the regeneration of ATP in a manner similar to that recently described by Yanari, Snoke, and Bloch (15) for glutathione synthesis.

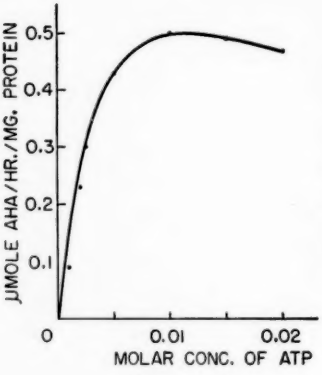


FIG. 3

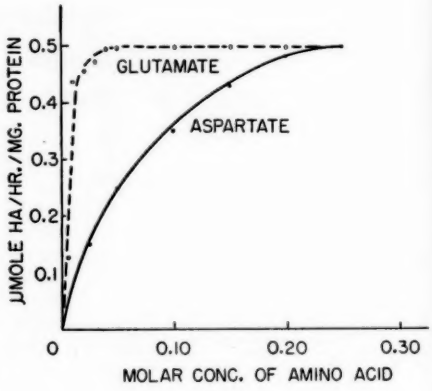


FIG. 4

FIG. 3. Dependence of aspartyl hydroxamate (AHA) synthesis on ATP concentration. The complete system contained 0.05 M Tris-HCl (pH 8.3), 0.10 M aspartate, 0.40 M hydroxylamine, 0.01 M $MgSO_4$, ATP in the concentrations given, and 0.5 ml. of an extract of wheat germ acetone powder in a total volume of 4 ml. Incubated at 38° for 60 minutes.

FIG. 4. Dependence of aspartyl hydroxamate (HA) synthesis on aspartate concentration contrasted with the dependence of glutamyl hydroxamate formation on glutamate concentration. The complete system contained 0.05 M Tris-HCl (pH 8.3), 0.40 M hydroxylamine, 0.01 M $MgSO_4$, 0.01 M ATP, aspartate in the concentrations given, and 0.5 ml. of an extract of wheat germ acetone powder in a total volume of 4 ml. Incubated at 38° for 60 minutes.

Properties of System Responsible for Aspartyl Hydroxamate Synthesis—In the light of the above evidence that asparagine is formed from aspartate in a manner at least somewhat analogous to the formation of glutamine from glutamate, the formation of aspartyl hydroxamate as described in Table I becomes of more interest. It seems possible that this reaction may be related to asparagine synthesis in the same way that glutamyl hydroxamate formation is related to glutamine synthesis. The properties of the aspartyl hydroxamate-forming system have, therefore, been examined as a prelude to purification of the enzyme which catalyzes asparagine synthesis. This makes it possible to establish in more detail how closely the properties of the aspartyl hydroxamate-forming system resemble those of the as-

paragine-forming system. As is evident from Fig. 3, the enzymatic formation of aspartyl hydroxamate, like the formation of glutamyl hydroxamate, has an absolute requirement for ATP. As might be expected, the completeness of this requirement is only apparent when either a well dialyzed extract or an extract of an acetone powder is used as the enzyme source. Neither adenosine diphosphate nor adenosine monophosphate is effective

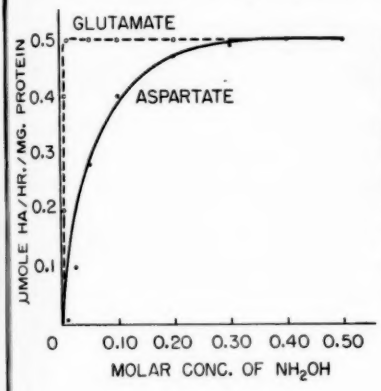


FIG. 5

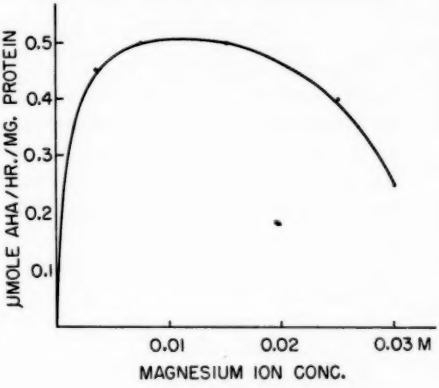


FIG. 6

FIG. 5. Dependence of aspartyl hydroxamate (HA) synthesis on hydroxylamine concentration contrasted with the dependence of glutamyl hydroxamate formation on hydroxylamine concentration. The complete system contained 0.05 M Tris-HCl (pH 8.3), 0.10 M aspartate, 0.01 M $MgSO_4$, 0.01 M ATP, hydroxylamine in the concentrations given, and 0.5 ml. of an extract of wheat germ acetone powder in a total volume of 4 ml. Incubated at 38° for 60 minutes.

FIG. 6. Dependence of aspartyl hydroxamate (AHA) synthesis on magnesium ion concentration. The complete system contained 0.05 M Tris-HCl (pH 8.3), 0.10 M aspartate, 0.01 M ATP, 0.40 M hydroxylamine, $MgSO_4$ in the concentrations given, and 0.5 ml. of an extract of wheat germ acetone powder in a total volume of 4 ml. Incubated at 38° for 60 minutes.

in replacing ATP. The pH optimum for aspartyl hydroxamate synthesis by wheat germ extracts is at pH 8.3, while the optimum for glutamyl hydroxamate synthesis is found around pH 7.4. The enzyme system has a relatively low affinity for aspartate. This is apparent from Fig. 4, in which the hydroxamate-forming activity of wheat germ extract is presented as a function of substrate concentration and compared with a similar curve for glutamyl hydroxamate formation. Even more striking are the differences in relative affinities of the two systems for hydroxylamine, as illustrated in Fig. 5. The exceedingly high affinity of the glutamine enzyme for hydroxylamine is not at all apparent in the aspartyl hydroxamate reaction.

Finally, aspartyl hydroxamate synthesis is highly sensitive to magnesium ion concentration (Fig. 6). Although aspartyl hydroxamate synthesis has an obvious absolute dependence on the presence of magnesium ions, it is also very sensitive to any increase in the concentration of these ions above 0.01 M. At Mg^{++} concentrations which are optimal for the synthesis of glutamine or glutamyl hydroxamate in plants, aspartyl hydroxamate synthesis is about 50 per cent inhibited.

DISCUSSION

There are striking differences in the properties of the enzymes responsible for the synthesis of glutamyl and aspartyl hydroxamates. Whether or not these differences are of importance to the manner of asparagine synthesis in the cell, and indeed whether the synthesis of aspartyl hydroxamate as studied here has any relation to asparagine synthesis itself, are questions that must await further investigation. The important facts are that aspartyl hydroxamate synthesis can be demonstrated and that there are considerable differences in the properties of the enzymes responsible for glutamyl and aspartyl hydroxamate synthesis. The differences are easily sufficient to explain the failure of previous investigators to detect the formation of aspartyl hydroxamate.

An important result of the present investigation has been the demonstration of a hitherto unreported pathway of asparagine synthesis; namely the direct amidation of aspartate in the presence of ATP in a manner that appears to be analogous to that involved in the synthesis of glutamine. Two other pathways of asparagine synthesis have been reported by other investigators. Mardashev and Lestrovaya (16) have asserted that liver extracts can form asparagine by a transamidation between glutamine and aspartate. In experiments with extracts of lupine and wheat germ, we have been unable to demonstrate any formation of radioactive asparagine from radioactive aspartate by such a reaction. It is somewhat doubtful, therefore, that the reaction occurs to any great extent in these plants. Meister and Fraser (17) have recently described the amination of α-ketosuccinamate to asparagine as catalyzed by liver preparations. It is not possible as yet to evaluate the importance of this reaction in the cellular synthesis of asparagine, because the biosynthesis of the amide bond of α-ketosuccinamate has not been demonstrated. It will be of definite interest to determine the relative importance of this reaction and the reaction described in the present paper in the general problem of asparagine synthesis in living cells.

SUMMARY

The synthesis of asparagine from aspartate and ammonia has been demonstrated in lupine seedlings and in extracts of these seedlings and of wheat

germ. In the latter case, ATP and magnesium ions are essential for the synthesis. The extracts readily catalyze the formation of aspartyl hydroxamate from aspartate, hydroxylamine, ATP, and magnesium ions. An investigation of the properties of this reaction indicates that the substrate relations, pH optimum, and sensitivity of the system to magnesium ion concentration are sufficiently different from those of the glutamine-forming system to explain the failure of previous investigators to detect aspartyl hydroxamate formation. The inability of at least some plant tissue extracts to form asparagine by the carboxylation of β-alanylamide or by a transamidation with glutamine has also been demonstrated.

BIBLIOGRAPHY

1. Elliott, W. H., *J. Biol. Chem.*, **201**, 661 (1953).
2. Webster, G. C., *Plant Physiol.*, **28**, 724 (1953).
3. Speck, J. F., *J. Biol. Chem.*, **179**, 1405 (1949).
4. Elliott, W. H., *Biochem. J.*, **49**, 106 (1951).
5. Elliott, W. H., and Gale, E. F., *Nature*, **161**, 129 (1948).
6. Grossowicz, N., Wainfan, E., Borek, E., and Waelsch, H., *J. Biol. Chem.*, **187**, 111 (1950).
7. Black, S., and Gray, N. M., *J. Am. Chem. Soc.*, **75**, 2271 (1953).
8. Schulze, E., *Z. physiol. Chem.*, **24**, 18 (1898).
9. Vickery, H. B., and Pucher, G. W., *J. Biol. Chem.*, **150**, 197 (1943).
10. Meiss, A. N., *Connecticut Agr. Exp. Sta., Bull. 553* (1952).
11. Webster, G. C., *Arch. Biochem. and Biophys.*, **47**, 241 (1953).
12. Webster, G. C., and Varner, J. E., *Arch. Biochem. and Biophys.*, in press.
13. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
14. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **195**, 669 (1952).
15. Yanâri, S., Snoke, J. E., and Bloch, K., *J. Biol. Chem.*, **201**, 561 (1953).
16. Mardashev, S. R., and Lestrovaya, N. N., *Doklady Akad. Nauk S. S. S. R.*, **78**, 547 (1951).
17. Meister, A., and Fraser, P. E., *J. Biol. Chem.*, **210**, 37 (1954).

THE AMINO ACID REQUIREMENTS OF MAN

XI. THE THREONINE AND METHIONINE REQUIREMENTS*

By WILLIAM C. ROSE, MINOR J. COON,† HAINES B. LOCKHART,‡ AND G. FREDERICK LAMBERT§

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois)

(Received for publication, December 20, 1954)

The minimal daily requirements of man for tryptophan (1), phenylalanine (2), and lysine (3) have been discussed in preceding papers of this series and *safe* intakes, designed to allow for variations in the needs of different individuals, have been proposed. The present paper extends these observations to threonine and methionine, and presents evidence regarding the minimal amounts of these amino acids which are capable of maintaining nitrogen equilibrium in adult males.

EXPERIMENTAL

The experimental procedures employed in all of the human investigations have been described in detail elsewhere (4, 1). As is our practice, healthy young men served as the subjects, and subsisted upon diets composed of mixtures of highly purified amino acids, wafers which furnished the major part of the energy and all of the inorganic salts, extra sucrose and butter fat as required in individual cases, and vitamins which were administered in the form of pills. The state of the nitrogen balance was taken as the criterion of dietary adequacy. The composition of the salt mixture, the baking powder used in making the wafers, and the vitamin pills will be found in Paper I of this series (4).

Three experiments, two of which are detailed below, were conducted in determining the threonine requirement. Subject R. L. B. received initially a diet composed of amino acid Mixture 104 (Table I), Wafers IV

* Aided by grants from the Nutrition Foundation, Inc., and the Graduate College Research Fund of the University of Illinois.

† Present address, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

‡ Some of the experimental data in this paper are taken from a thesis submitted by Haines B. Lockhart in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois. Present address, Research Laboratories, Swift and Company, Chicago, Illinois.

§ Present address, Nutrition Research Department, Abbott Laboratories, North Chicago, Illinois.

XUM

(the composition of which has been described in an earlier paper (5)), 141.4 gm. of extra sucrose, and 15.0 gm. of extra butter fat. As shown in Table I, amino acid Mixture 104 contained 0.20 gm. of L-tryptophan and 0.75 gm. of L-lysine monohydrochloride. These quantities, though smaller than those administered in later experiments, were known to be sufficient to meet the needs of Subject R. L. B., inasmuch as he had served in one of the lysine tests (*cf.* (3)). The daily allotment of amino acid Mixture 104

TABLE I

Composition and Daily Intakes of Amino Acid Mixtures

Component	Mixture 104		Mixture 164		Mixture 172	
	As used	N content	As used	N content	As used	N content
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Valine	2.40*	0.29	3.20*	0.38	3.20*	0.38
Leucine	4.91	0.52	10.14*	1.08	4.91	0.52
Isoleucine	6.16*	0.66	6.36*	0.68	6.16*	0.66
Methionine	3.68*	0.35	3.80*	0.36	3.68*	0.35
Threonine	1.20*	0.14	1.20*	0.14	1.60*	0.19
Phenylalanine	3.00*	0.25	2.40*	0.20	2.40*	0.20
Tryptophan	0.20	0.03	0.30	0.04	0.30	0.04
Lysine monohydrochloride	0.75	0.12	2.00	0.31	2.00	0.31
Glycine	29.10	5.43	25.72	4.80	27.54	5.14
Urea	4.29	2.00	4.43	2.06	4.29	2.00
	55.69	9.79†	59.55	10.05‡	56.08	9.79§

* Racemic acids.

† Of the nitrogen contributed by this mixture, 0.54 gm. was derived from the D forms of valine, isoleucine, and threonine.

‡ Of the nitrogen contributed by this mixture, 1.14 gm. were derived from the D forms of valine, leucine, isoleucine, and threonine.

§ Of the nitrogen contributed by this mixture, 0.61 gm. was derived from the D forms of valine, isoleucine, and threonine.

furnished 9.79 gm. of nitrogen, of which 0.54 gm. was derived from the D isomers of valine, isoleucine, and threonine. The total nitrogen content of the diet was 10.10 gm., of which 0.31 gm. was of an unknown nature, and had its origin chiefly in the starch of the wafers and in lesser amounts in the butter fat, lemon juice used as a flavoring agent for the amino acid solution, and the liver concentrate present in the vitamin pills.

Subject E. E. W. received initially a diet consisting of amino acid Mixture 164 (Table I), Wafers IV, 242.7 gm. of extra sucrose, and 32.6 gm. of extra butter fat. Mixture 164 contained DL-leucine instead of the L-amino acid, since a supply of the latter known to be free of methionine and iso-

leucine was temporarily exhausted. The daily allotment of Mixture 164 furnished 10.05 gm. of nitrogen, of which 1.14 gm. were derived from the D forms of valine, leucine, isoleucine, and threonine. The total nitrogen content of the diet of Subject E. E. W. was 10.37 gm., of which 0.32 gm. was of an unknown nature. In each experiment, Cellu flour, to provide a residue in the alimentary tract, was administered apart from the basal rations. The daily energy intake of the subjects amounted to 55 calories

TABLE II

Threonine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
			Subject R. L. B.			
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
10	64.9	10.10	9.35	0.49	+0.26	1.2 gm. DL-threonine
7	65.3	10.10	9.17	0.57	+0.36	0.6 " "
5	65.3	10.10	8.94	0.58	+0.58	0.8 " "
5	65.8	10.10	9.61	0.52	−0.03	0.4 " "
4	65.8	10.10	10.42	0.64	−0.96	0.8 " D-threonine
			Subject E. E. W.			
5	77.1	10.37	9.25	0.82	+0.30	1.2 gm. DL-threonine
5	78.0	10.37	9.78	0.91	−0.32	0.6 " "
7	77.6	10.37	9.76	0.76	−0.15	0.4 " L-threonine
4	78.9	10.37	9.05	0.73	+0.59	0.6 " "
5	79.4	10.37	9.24	0.82	+0.31	0.5 " "

* The initial diets of Subjects R. L. B. and E. E. W. contained amino acid Mixtures 104 and 164, respectively.

per kilo of body weight, and the ratio of calories derived from carbohydrates to those furnished by fats was maintained at 2.6. One-third of the amino acid solution and other dietary components was consumed at each meal.

The results of the two threonine experiments are summarized in Table II. In the case of Subject R. L. B., DL-threonine was used in measuring his minimal requirement. The data for the first two periods show that daily intakes of 1.2 and 0.6 gm., respectively, of the racemic amino acid induced positive nitrogen balances. During the third period, the dosage was raised to 0.8 gm. This seems unnecessary in view of the findings of the preceding period, and requires an explanation. As a matter of fact, in

nitrogen balance investigations one cannot always be certain as to the outcome of a test, even on the final day of a period, since the feces do not become available for analysis until approximately 24 hours after the urine collection has been completed. With Subject R. L. B., a test at the 0.8 gm. level of DL-threonine was not needed since 0.6 gm. daily had proved to be sufficient; however, the adequacy of the lower intake could not be predicted with certainty at the time the dietary alteration was made. During the penultimate period, the daily administration of 0.4 gm. of DL-threonine led to an average daily loss of 0.03 gm. of nitrogen. Negative nitrogen balances occurred on all days of the period except the first, when the higher threonine intake of the preceding period evidently was responsible for a moderately positive balance.

During the final period of the experiment, the daily consumption of 0.8 gm. of D-threonine resulted in an average daily loss of 0.96 gm. of nitrogen. In another test (Subject H. B. L.), the details of which are omitted, the daily administration of 0.8 gm. of D-threonine induced an average daily loss of 1.18 gm. of nitrogen. Negative balances of this magnitude are comparable to those observed when the diets are devoid of threonine (*cf.* (6)). Consequently, if any utilization of D-threonine occurs in man, the amount is too small to be detected by the nitrogen balance technique. Furthermore, one must conclude from the findings in the experiment upon Subject R. L. B. that his minimal daily requirement was 0.6 gm. of DL-threonine, or 0.3 gm. of L-threonine. With most amino acids, no attempt has been made to establish the minimal needs with greater precision than that represented by 0.1 gm. of the L isomer. To do so, even if feasible, would be unprofitable in view of the variations in requirements observed from one individual to another.

The experiment upon Subject E. E. W. (Table II) illustrates the individual inconstancy mentioned above. This young man came into positive nitrogen balance with a daily intake of 1.2 gm. of DL-threonine, but, in contrast to the behavior of Subject R. L. B., experienced a distinct loss of nitrogen when the daily dosage was reduced to 0.6 gm. At this point in the experiment, DL-threonine was replaced by the L isomer at a level of 0.4 gm. daily. This also led to a negative nitrogen balance. During the last two periods, the daily intake was first raised to 0.6 gm. and then lowered to 0.5 gm. Both dosages proved to be adequate. From these data it is clear that the minimal daily L-threonine requirement of E. E. W. was 0.5 gm. A third experiment (Subject H. B. L.), the tabular data for which are omitted to conserve space, was conducted with DL-threonine. The results indicated that 1.0 gm. of the racemic amino acid was the smallest amount which could induce a positive balance. As pointed out above, D-threonine was totally ineffective in this subject; consequently, his minimal daily L-threonine requirement was 0.5 gm.

On the basis of the above results, the threonine content of diets used in investigating other amino acids was reduced to a level which provided only a moderate excess over the apparent minimal requirement (*cf.* (1)). Thus, four subjects received rations containing 0.6 gm. daily of L-threonine (administered as 1.2 gm. of the racemic amino acid) and were maintained in positive nitrogen balance. Later, sixteen additional subjects received daily doses of 0.8 gm. of L-threonine, in the form of the racemic acid, and all showed positive nitrogen balances. No attempt was made to determine the exact minimal requirements of these twenty individuals, but obviously their maintenance needs could not have exceeded the quantities of threonine which they actually consumed.

In accordance with the practice followed throughout these studies, the highest observed figure, *i.e.* 0.5 gm. of L-threonine or twice this amount of DL-threonine, is hereby proposed *tentatively* as the minimal daily requirement of adult males. Furthermore, in the light of our experience, 1.0 gm. of L-threonine certainly may be regarded as a *safe* daily intake. Indeed, it is extremely improbable that any normal, human subject would have a minimal requirement in excess of this quantity. To date, twenty-six different individuals have been maintained in positive nitrogen balance on daily intakes of 2.0 gm. or less of DL-threonine, and no one has been found with a higher requirement.

Six experiments have been carried out in establishing the minimal daily methionine requirement. The results of two such tests are presented in summary form in Table III. Both subjects (G. W. L. and E. E. W.) received initially diets containing amino acid Mixture 172 (Table I), Wafers IV, and the customary vitamin pills. The rations differed only in the amounts of extra sucrose and butter fat necessary to furnish to each subject a daily energy intake of 55 calories per kilo of body weight. For this purpose, Subject G. W. L. received 129.8 gm. of extra sucrose and 14.3 gm. of extra butter fat. The corresponding quantities consumed by Subject E. E. W. were 207.4 and 26.9 gm. As usual, the ratio of calories derived from carbohydrates to those from fats was maintained at 2.6. Amino acid Mixture 172 furnished 9.79 gm. of nitrogen daily, of which 0.61 gm. was derived from the D forms of valine, isoleucine, and threonine. The daily nitrogen intakes from all sources were 10.10 gm. for Subject G. W. L. and 10.11 gm. for Subject E. E. W. Of these quantities, 0.31 and 0.32 gm., respectively, were of an unknown nature, the slight difference being due to the somewhat larger consumption of extra butter fat by E. E. W.

As the figures in Table III demonstrate, the findings in the two tests were almost identical throughout. At the start, each subject received 3.68 gm. of DL-methionine daily, the amount which had been used routinely in most of the preceding quantitative investigations (1–3). With this régime, the young men showed positive nitrogen balances as anticipated.

During the second period of each experiment the DL-methionine intake was reduced to 0.60 gm., with the result that slightly negative balances occurred. During the third period, with dosages of 0.80 gm. per day, almost exact balance was observed in each case. One is tempted, therefore, to

TABLE III

Methionine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
			Subject G. W. L.			
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
6	65.8	10.10	9.09	0.72	+0.29	3.68 gm. DL-methionine
8	66.7	10.10	9.50	0.68	−0.08	0.60 " "
9	66.7	10.10	9.25	0.81	+0.04†	0.80 " "
7	66.7	10.10	8.91	0.71	+0.48	1.00 " "
6	66.2	10.10	9.14	0.75	+0.21	0.90 " "
6	66.7	10.10	9.22	0.83	+0.05	1.00 " D-methionine
6	67.6	10.10	9.45	0.70	−0.05	0.80 " "
			Subject E. E. W.			
9	73.5	10.11	9.12	0.74	+0.25	3.68 gm. DL-methionine
8	73.9	10.11	9.42	0.76	−0.07	0.60 " "
7	74.8	10.11	9.35	0.75	+0.01‡	0.80 " "
8	74.8	10.11	9.28	0.69	+0.14	0.90 " "
5	74.8	10.11	8.87	0.58	+0.66	1.00 " "
6	75.8	10.11	9.06	0.61	+0.44	1.00 " D-methionine
5	76.2	10.11	9.47	0.65	−0.01	0.80 " "

* The initial diets of Subjects G. W. L. and E. E. W. contained amino acid Mixture 172.

† Subject G. W. L. was in positive balance during 4 days and in negative balance during 5 days of this period.

‡ Subject E. E. W. was in positive balance during 2 days and in negative balance during 5 days of this period.

regard 0.80 gm. as representing the minimal needs of the subjects. Indeed, such a conclusion would not be greatly in error. However, as stated in foot-notes to Table III, Subject G. W. L. was in positive nitrogen balance during only 4 of the 9 days of the period, and Subject E. E. W. was in positive balance during only 2 of the 7 days of the period. In instances of this sort, as mentioned previously (1, 2), the practice has been followed of designating as the minimal requirement the smallest quantity

of an amino acid which is capable of inducing a *distinctly* positive balance as measured by the average of a period of several days. In accordance with this principle, 0.90 gm. daily, which was consumed during the fifth and fourth periods, respectively, of the two experiments, must be accepted as the DL-methionine requirement of the subjects.

Of unusual interest are the results obtained during the last two periods of the experiments upon Subjects G. W. L. and E. E. W. (Table III), when the metabolic availability of D-methionine was tested at two levels of intake. Daily dosages of 1.00 and 0.80 gm. were chosen as the most appropriate, since these quantities were slightly greater and slightly less, respectively, than the minimal DL-methionine requirement of the subjects, and since the effects of like intakes of the racemic amino acid had already been determined. Examination of the data reveals the fact that D-methionine was almost, if not quite, as effective as DL-methionine. Indeed, the slight differences shown are believed to be well within the limits of accuracy of the nitrogen balance technique.

Inasmuch as D- and DL-methionine are virtually equal in metabolic behavior, it follows that L-methionine cannot possess a measurably greater activity. Consequently, no significant error is introduced if figures representing the minimal requirements, as determined by the use of the racemic amino acid, are regarded as identical with values which would have been obtained if L-methionine had been employed as the test compound. Such an assumption has been made in summarizing the results of the six methionine experiments which are presented, together with the three threonine tests, in Table IV. The similarity of the methionine figures and those representing the L-phenylalanine requirements, as described in Paper IX (2) of this series, is most striking. Even the range of values (0.80 to 1.10 gm.) is identical for the two amino acids. However, one should recall that this fortuitous parallelism was disclosed by the use of diets which were devoid of cystine and tyrosine, and that the similarity might become less apparent if these amino acids were present in the food. Later papers will demonstrate that cystine and tyrosine exert marked sparing effects upon the requirements of the human organism for methionine and phenylalanine, respectively.

In accordance with the practice followed with other essential amino acids, and for the reasons discussed elsewhere (1, 2), the highest observed value, or 1.10 gm. daily, is proposed as the *tentative* L- or DL-methionine requirement of adult human males. To make liberal allowance for the possibility that individuals other than those tested may have higher requirements, twice this tentative minimum, or 2.20 gm. of L- or DL-methionine, may be regarded as a *safe* daily intake. In the future, experience may warrant a lowering of this quantity without incurring the danger of a

deficiency. To date, twenty individuals have been maintained in positive nitrogen balance on daily intakes of 2.20 gm. or less. Of these, seventeen received 2.00 gm. or less. In no case did even the lower level prove to be inadequate.

TABLE IV

Summary of Threonine and Methionine Experiments

Subject	Body weight	Minimal daily requirement
	L-Threonine requirement*	
	kg.	*gm.*
H. B. L.	59.4	0.5
R. L. B.	64.9	0.3
E. E. W.	77.1	0.5
	L-Methionine requirement†	
A. A. S.	59.8	1.0
G. W. L.	65.8	0.9
H. S. E.	72.6	1.1
E. E. W.‡	73.5	0.9
A. G.	75.0	0.8
R. L. W.	81.9	0.9

* DL-Threonine was used in measuring the requirements of two of the three subjects but, since D-threonine is entirely ineffective, the minimal needs are expressed in terms of the L-amino acid.

† DL-Methionine was used in measuring the requirements of all subjects but, since the D and L isomers are virtually equal in effectiveness, in so far as one can determine by the nitrogen balance technique, the minimal needs are expressed in terms of the L-amino acid.

‡ This subject served in both the threonine and methionine tests. During the intervening 7 months he reduced his body weight by 3.6 kilos.

The data in Table IV are in accord with the conclusion reached in earlier papers (1–3) to the effect that no observable relationship exists between the minimal amino acid requirements of subjects and their body weights. Calculations also reveal the absence of correlation between minimal needs and body surface or creatinine output.

SUMMARY

By making use of the techniques customarily employed in this laboratory, experiments have been conducted upon normal young men for the purpose of establishing their minimal threonine and methionine require-

ments. As with the amino acids previously considered, the findings varied somewhat from one individual to another but appeared to be independent of body weight, body surface, or creatinine output.

Three experiments were carried out with threonine. In two of these, DL-threonine was the test compound. The results showed that the subjects required daily intakes of 0.6 and 1.0 gm., respectively. In the third experiment, L-threonine was used. With this, the minimal requirement of the subject was 0.5 gm. daily. D-Threonine was found to be devoid of measurable influence upon nitrogen balance. In view of this fact, the minimal daily requirements of the three subjects may be expressed as 0.3, 0.5, and 0.5 gm., respectively, of L-threonine. In conformity with the practice followed with other essential amino acids, it is proposed that the highest observed finding, or 0.5 gm. of L-threonine daily, be designated *tentatively* as the minimal daily requirement of man.

In formulating diets designed to exclude amino acid deficiencies, allowance should be made for the possibility that individuals other than those tested may require still larger intakes of threonine. For this purpose, 1.0 gm. of the L isomer, or twice this amount of the racemic compound, is certainly a *safe* daily intake. To date, twenty-six young men have been maintained in positive nitrogen balance upon 2.0 gm. or less of DL-threonine per day. Indeed, four of these subjects received 1.2 gm. and sixteen consumed 1.6 gm. daily of the racemic acid, though no attempt was made to establish their exact minimal needs.

Six experiments were carried out in determining the minimal methionine requirement. The results demonstrate that the subjects required daily intakes of 0.8, 0.9, 0.9, 0.9, 1.0, and 1.1 gm., respectively, of the racemic amino acid. D-Methionine, in contrast to the behavior in man of the D isomers of all other essential amino acids, proved to be as effective as DL-methionine in the maintenance of nitrogen equilibrium. From this it follows that the L isomer does not possess greater metabolic activity than does the racemic compound, and that the methionine requirement is the same whether expressed in terms of the L- or the DL-amino acid.

Because of considerations discussed in earlier papers, the highest observed value, or 1.1 gm., is designated *tentatively* as the minimal daily methionine requirement of man. Twice this quantity, or 2.2 gm. of either the L- or the DL-amino acid, is a *safe* daily intake. To date, twenty individuals have been maintained in positive nitrogen balance upon this amount, and no subject has been found who could not be so maintained.

BIBLIOGRAPHY

1. Rose, W. C., Lambert, G. F., and Coon, M. J., *J. Biol. Chem.*, **211**, 815 (1954).
2. Rose, W. C., Leach, B. E., Coon, M. J., and Lambert, G. F., *J. Biol. Chem.*, **213**, 913 (1955).

3. Rose, W. C., Borman, A., Coon, M. J., and Lambert, G. F., *J. Biol. Chem.*, **214**, 579 (1955).
4. Rose, W. C., Johnson, J. E., and Haines, W. J., *J. Biol. Chem.*, **182**, 541 (1950).
5. Rose, W. C., Coon, M. J., and Lambert, G. F., *J. Biol. Chem.*, **210**, 331 (1954).
6. Rose, W. C., Haines, W. J., Warner, D. T., and Johnson, J. E., *J. Biol. Chem.*, **188**, 49 (1951).

FRUCTOSE-AMINO ACIDS IN LIVER: STIMULI OF AMINO ACID INCORPORATION IN VITRO*

By HENRY BORSOOK, ADOLPH ABRAMS,† and PETER H. LOWY

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California)

(Received for publication, October 18, 1954)

We have reported that the incorporation *in vitro* of amino acids into proteins of rabbit reticulocytes was stimulated by a boiling water extract of commercially dried liver or by the filtrate of boiled plasma. Certain amino acids, *i.e.* histidine, leucine, phenylalanine, and valine, had a stimulating effect. The liver and plasma extracts certainly contained these amino acids, but even optimal concentrations could account for less than half the stimulating action of the extracts; indeed the amino acids were synergistic with the extracts. Practically all the familiar metabolites, vitamins, and cofactors were tested; none could account for the stimulating effects of the liver extract or of boiled plasma filtrate (1).

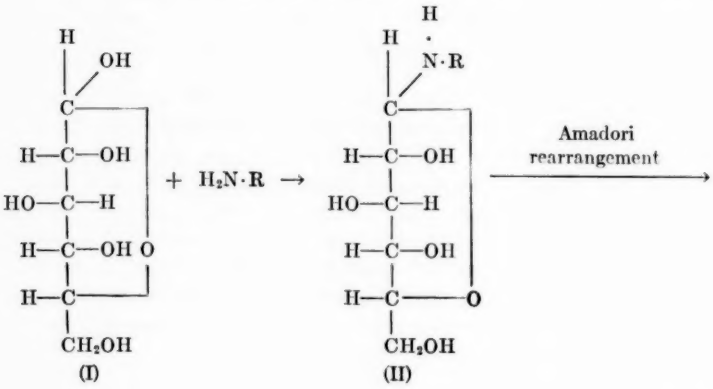
Most of the stimulating activity of the liver extract we have used can be accounted for by the above amino acids acting in conjunction with iron and a class of substances not hitherto identified in animal or plant tissues. These substances are 1-deoxy-1-(*N*-amino acid)-2-ketohexoses. They can be viewed as formed from the corresponding *N*-glucosides of the amino acids by Amadori rearrangement (2–5).

It is not established whether the sugar residue, after the rearrangement, forms a furanose or pyranose ring. Compounds of the type of III have been designated, according to Fischer (6), as *N*-substituted isoglucosamines. When R is an amino acid residue, III gives a positive, weak test with the Elson-Morgan color reagent for glucosamine (7). The keto group at C-2 is responsible for the characteristic ability of these compounds to reduce in 0.1 N NaOH oxidation-reduction dyes (*e.g.*, methylene blue or 2,6-dichlorophenolindophenol) or ferricyanide rapidly at room temperature.

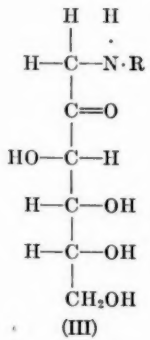
* These studies were aided by a contract between the Atomic Energy Commission, administered by the Office of Naval Research, Department of the Navy, and the California Institute of Technology, Division of Biology (NR-122-107). They were also supported by a research grant from the National Institutes of Health, United States Public Health Service, and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

† Supported by a grant from Eli Lilly and Company. Present address, Department of Biochemistry, Medical Center, University of Colorado, Denver, Colorado.

It can be seen that the same Amadori rearranged product is obtained when the parent sugar is either glucose or mannose; the sugar radical after the rearrangement has the configuration of a fructose compound; analogously galactose gives a tagatose compound. The completely descriptive names are cumbersome; for convenience they will be designated here as



(I) (II)



(III)

fructose- (or tagatose-)alanine (or glycine, etc.) and abbreviated F-Ala, F-Gly, T-Ala, T-Gly, etc., according to the configuration of the sugar residue after the Amadori rearrangement and the constituent amino acid. The Amadori rearrangement occurs slowly in the solid state at 25° and rapidly in hot alcoholic solutions.

We report here the method which we have used to isolate fructose-amino acids from liver, some of the characteristic chemical and physical properties of these substances, and their stimulation of amino acid incorporation *in vitro* into the proteins of rabbit reticulocytes.

Procedure

The production and washing of the reticulocytes, the preparation of the C^{14}-amino acids, the saline solutions, and the reaction mixture which contained the stimulating amino acid mixture were as described previously (1). The incubation time was 4 hours.

We have modified somewhat the procedure of washing the proteins to remove free labeled amino acid. At the end of the incubation the contents of each beaker were added to 80 ml. of water and the reticulocyte protein precipitated with 20 ml. of 35 per cent trichloroacetic acid. When the protein had settled well, usually after 3 hours, the clear supernatant solution was decanted and drained off, and the protein dissolved in 2.5 ml. of 1 N NaOH and then reprecipitated with 100 ml. of 7 per cent trichloroacetic acid. On the next day the precipitate was washed, by centrifugation, five times with 7 per cent trichloroacetic acid, twice with a mixture of equal volumes of acetone and ether, twice with acetone, and finally twice with ether. The proteins were dried for 1 hour at 85°, and their radioactivity was measured as described previously (8).

Reduction Test—A simple and rapid test to follow isolation of the ketose-amino acids in liver or of the synthetic compounds from a reaction mixture was devised, based on reduction at room temperature of ferricyanide and conversion of the ferrocyanide to Prussian blue.

Usually we have used an aliquot of 0.1 ml. To this was added 1 ml. of 0.1 per cent potassium ferricyanide in water, followed by enough 1 N NaOH to make the solution 0.1 N, not counting the alkali required, which was determined beforehand, to bring the pH to the turning point with phenolphthalein. The reaction was allowed to proceed at room temperature for exactly 5 minutes, after which time was added 1 ml. of a ferric sulfate solution prepared according to Folin and Malmros (9). After another 5 minutes, 5 ml. of water were added. The blue color was then measured at 690 mμ.

The aliquot must be so adjusted that the ferricyanide is not completely reduced. For quantitative comparisons the time from the addition of alkali to the addition of ferric sulfate is critical, the longer the time the more reduction. We have chosen a reaction time of 5 minutes because in this time there is a linear relation between the amount of blue color finally obtained and the amount of ketose-amino acid up to 0.2 mg. One can measure by the above procedure 0.01 mg. and detect 0.002 mg.

Ascorbic acid, creatinine, cysteine, glutathione, and tyrosine react positively to this test and need to be taken into account if one uses the above procedure to assay crude extracts of biological material. Glucose gives about 2 per cent of the reduction of an equivalent weight of ketose-amino acids.

Reduction Test for Ketose-Amino Acids on Paper Chromatograms—0.1 per cent potassium ferricyanide was made up in 80 per cent ethanol; not all the ferricyanide went into solution. Just before use one-tenth its volume of 10 per cent NaOH in 80 per cent ethanol was added to it, which brought all the ferricyanide into solution. The alkaline ferricyanide solution was sprayed on the paper lightly. After 5 minutes the ferric reagent (as above) was sprayed on lightly. A positive test was a dark blue spot against a colorless or light blue background. The color was stable. It should be noted that there was no heating. 10 γ were detected easily.

Ninhydrin Test—Paper chromatograms were dipped in 1 per cent ninhydrin in pyridine and drained, and the color was then developed quickly under a heating lamp or more slowly for $\frac{1}{2}$ to 1 hour at room temperature.

Determination of Total Sugar—Total sugar was determined by three methods, with carbazole in Dische's procedure (10, 11), Dreywood's anthrone reagent (12), and hot reduction of ferricyanide in alkaline solution by the procedure of Park and Johnson (13), except that instead of their ferric solution containing Duponol, to keep the Prussian blue suspended, we have used Folin and Malmros' (9) solution containing gum ghatti. The first two of these methods depend on formation of a colored product by condensation with either anthrone or carbazole of a furfural derivative formed from the sugar by concentrated sulfuric acid; the results obtained by either of these methods and by hot reduction of ferricyanide in alkali are markedly different and indicative of whether the sugar is a simple glucoside or conjugated as in the fructose-amino acids.

Glucosamine Color Reaction—After glucosamine is heated in 0.25 N Na_2CO_3 with acetylacetone, it gives with *p*-dimethylbenzaldehyde a purple compound. Fructose-amino acids react positively to this test, but the color per equivalent is much lighter than that yielded by glucosamine. The procedure of Elson and Morgan (7) was followed exactly, except that the color was read in a spectrophotometer at 550 mμ, which is the region of maximal absorption.

Determination of Acid and Basic Groups—The acid and basic groups were determined by electrometric titration in a CO_2-free atmosphere with a glass electrode, the acid groups in 90 per cent acetone, the basic groups in water.

Isolation of Fructose-Amino Acids from Liver—500 gm. of dried hog liver powder[1] were suspended in 2 liters of 7 per cent trichloroacetic acid, stirred slowly at room temperature for 2 hours, and then filtered with the aid of Super-Cel through a No. 50 Whatman paper with suction. The filter cake was suspended in 500 ml. of 7 per cent trichloroacetic acid, stirred thoroughly, and again filtered. Both filtrates were combined, and then an equal volume of 10 per cent $Pb\cdot(C_2H_3O_2)\cdot 3H_2O$ in aqueous acetic acid

[1] Hog liver, dried at 75°, obtained from the Viobin Laboratories, Monticello, Illinois.

(pH 4.0) was added. After standing for 3 hours, the suspension was filtered and the precipitate was washed with water until the filtrate was chloride-free. The washed precipitate (after removal of the lead) had no stimulating activity in our test system. The combined filtrate and washings were brought to pH 9.5 with 5 N NaOH and allowed to stand for about 3 hours, when the precipitate had settled, leaving about half the total volume of the suspension as a clear supernatant solution. Partly by decantation and finally by centrifugation the precipitate was collected in 100 ml. centrifuge tubes and washed by repeated suspension in water and recentrifugation. The supernatant solutions were discarded. A stage is reached in the washing of the precipitate when, even after prolonged centrifugation, the supernatant solution remains turbid. At this stage the latter was very nearly chloride-free. The precipitate washed to this point was suspended in about 200 ml. of water and brought into solution with glacial acetic acid, final pH about 4.5. The lead was removed with H_2S, and, after removing the excess H_2S by aeration, the filtrate from the lead sulfide was lyophilized. Usually the residue weighed about 12 gm.; it was pale yellow and somewhat hygroscopic.

The next stage in the purification was chromatography on Dowex 50. The resin was cleaned by the procedure of Moore and Stein (14) and used in the H^+ form. A column of resin 22 × 450 mm. was washed with redistilled water until the eluate gave a negative test with $AgNO_3$ and the pH was above 5. Then 6 gm. of the material recovered from the lead precipitation, dissolved in 10 ml. of water, were washed into the column with water, and 20 hold-up volumes of water were passed through at a rate of 1 hold-up volume per hour. Then 20 hold-up volumes of each of the following strengths of trichloroacetic acid were passed through in succession: 0.2, 0.5, 0.75, and 1.0 M. The trichloroacetic acid eluates were collected in 0.5 hold-up volume fractions.

In exploratory experiments it had been found that stimulating activity in our test system paralleled the intensity of reduction of ferricyanide (method described above). Accordingly, this test was made the basis of the chromatographic separation. 0.1 to 0.3 ml. aliquots of each fraction were taken for the test, according to the intensity of the reduction obtained. The volumes of the reagents used were the same for 0.1 to 0.3 ml. aliquots except for the additional alkali needed for neutralization. Fig. 1 is a typical chromatogram.

The fractions constituting each band in Fig. 1 were pooled and lyophilized after removal of the trichloroacetic acid with ether. The material in every one of these bands stimulated the incorporation of leucine *in vitro* into rabbit reticulocyte proteins; the eluates between the bands had no stimulating action; there was some stimulating material in the water eluate.

So far only bands I, II, and III have been worked up further. They

were rechromatographed twice on the same resin in columns of the same dimensions, and the bands were located as before by the reduction test. 20 hold-up volumes of water were passed through and discarded. Bands I and II were eluted with 0.05 M and band III with 0.1 M trichloroacetic acid after 20 hold-up volumes of the 0.05 M solution had passed through. After each chromatography the trichloroacetic acid was extracted with ether, and the solution was then lyophilized. After the second chromatography the dried residue was dissolved in absolute methanol, from which it was precipitated by the addition of 10 volumes of dried ether. The solution in methanol and precipitation by ether were repeated twice more.

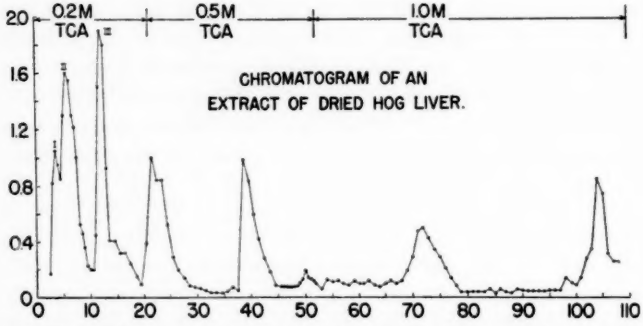


FIG. 1. Chromatogram of an extract of dried hog liver. Resin, Dowex 50 H^+; column dimensions, 22 × 450 mm.; eluent, varying strengths of trichloroacetic acid; material chromatographed, trichloroacetic extract of dried hog liver and material carried down with lead hydroxide on bringing lead acetate solution to pH 9.5. Ordinates, reduction values in terms of optical density of ferriferrocyanide formed; abscissas, hold-up volumes.

This procedure yielded, in the case of band II, a single component, fructose-L-glutamic acid. Both bands I and III were mixtures of fructose-alanine and fructose-glycine; the proportion and mode of linkage of the sugar were different in the two bands, but in each case the components of the mixture had very similar chromatographic properties. 500 gm. of dried liver usually gave 75, 250, and 200 mg., respectively, of compounds corresponding to bands I, II and III.

The composition of bands II and III was proved by comparison with corresponding synthetic compounds (for the nature of the material in band I see below). The details of the synthesis will be given in a later communication.

Sugar-protein compounds are formed slowly during storage of dried foods, and there is evidence (15) that they are of a type that can and does undergo

Amadori rearrangement to compounds of the above type III. The material in the bands of Fig. 1 may have been formed during and after the drying of the liver or in the isolation procedure. To test this point a hog liver was obtained directly from the animal, immediately frozen with solid CO_2, minced at 0°, and then precipitated with 7 per cent trichloroacetic acid at 0°. From this point on the procedure was identical with that described above. The chromatography of the fresh differed from that of the dried hog liver in that 0.35 M trichloroacetic acid was interposed before and 0.75 M

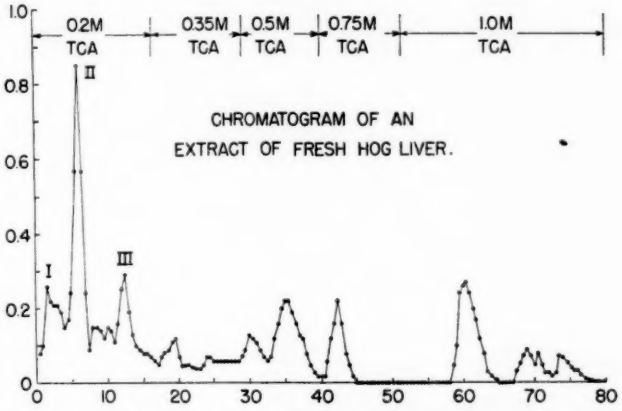


FIG. 2. Chromatogram of an extract of fresh hog liver. Resin, Dowex 50 H^+; column dimensions, 22 × 450 mm.; eluent, varying strengths of trichloroacetic acid; material chromatographed, cold trichloroacetic acid extract of fresh hog liver and material carried down with lead hydroxide on bringing lead acetate solution to pH 9.5. Ordinates, reduction values in terms of optical density of ferriferrocyanide formed; abscissas, hold-up volumes.

after the 0.5 M acid. The chromatogram, Fig. 2, is seen to be qualitatively similar to that given by the dried hog liver, Fig. 1. Bands II and III from fresh and from dried liver proved to be the same material. There was too little of band I from fresh liver to characterize it as extensively as the corresponding band from dried liver.

Comparison of Band II Material with Synthetic Fructose-L-Glutamic Acid—Table I shows that fructose-L-glutamic acid and the material in band II (Figs. 1 and 2) from both dried and fresh hog liver were the same.

The hydrolysis was carried out in 1.0 N HCl at 100° for 2 hours. With all three preparations the yield of amino acid was much less than half the theoretical. The solutions became dark brown, almost black. Both the

low yield of amino acid and the intense browning on heating with strong acid are characteristic of fructose-amino acids.

The difference in optical rotation of the two preparations recorded in Table I is within the experimental error of the micromethod used.

It is characteristic of all the fructose- and tagatose-amino acids we have prepared that they yield low values for sugar by methods based on conversion to furfural derivatives, as in the anthrone (12) and carbazole methods (10, 11), and nearly theoretical values by methods based on their reducing

TABLE I

Comparison of Chemical and Physical Properties of Synthetic Fructose-L-Glutamic Acid with Band II Material from Dried and Fresh Hog Liver

	Synthetic	Dried liver	Fresh liver
Amino acid released on acid hydrolysis	Glutamic	Glutamic	Glutamic
Total N, theoretical, %	4.53	4.53	
" " observed, %	4.67	4.40	
$[\alpha]_D^{25}$ (H_2O), degrees	−40 (*c* 3.27) −38 (*c* 6.32)	−34 (*c* 6.55)	
No. of titratable groups, acid	2	2	
" " " " basic	1	1	
pK′ of basic group	8.1–8.6	8.1–8.6	
Glucose, theoretical, equiv. per mole	1.00	1.00	1.00
Determined by anthrone	0.39	0.42	0.37
" " carbazole	0.72	0.55	0.85
" " hot reduction of ferricyanide	0.91	0.82	0.87
Reduction, % of that by equivalent amount of synthetic fructose-glycine	106	104	109
Glucosamine color, % of that given by equivalent weight of glucosamine	3.8	5.0	4.9
Paper chromatography			
Butanol-acetic acid-water, R_F	0.10, 0.22	0.10, 0.22	0.11, 0.22
Propanol-ammonia, R_F	0.095, 0.24	0.082, 0.22	0.086, 0.22

property, as in the hot reduction of ferricyanide. This is shown in Table I.

In Table I and throughout this communication the reduction values obtained at room temperature were rates measured under standarized conditions, as described above, and compared with the reduction under the same conditions by an equivalent amount of synthetic fructose-glycine.

With the glucosamine color reagent, fructose-glutamic acid gave, under the conditions employed, about 5 per cent of the color produced by an equivalent weight of glucosamine. A positive but low value by this test is characteristic of fructose-amino acids. Gottschalk (16) reported a value for fructose-DL-phenylalanine as 10 per cent that of glucosamine, which is the same as that of fructose-alanine and fructose-glycine (see Table II).

In the filter paper chromatography a mixture of butanol-acetic acid-water in the proportion of 4:1:1 and of propanol-ammonia (1 per cent), 3:1, was used. The chromatograms were developed with both the ninhydrin and reduction reagents. The synthetic fructose-L-glutamic acid and those isolated from dried and from fresh liver all gave two reducing spots with cold reagents, but the material of only one of these spots, the compound with

TABLE II

Comparison of Chemical and Physical Properties of Synthetic Fructose-L-Alanine and Fructose-Glycine with Band III Material from Dried and Fresh Hog Liver

	Synthetic		Band III	
	F-Ala	F-Gly	Dried liver	Fresh liver
Amino acid released on acid hydrolysis	Alanine	Glycine	Alanine, glycine	Alanine, glycine
Total N, theoretical, %	5.58	5.92	5.58–5.92	5.58–5.92
" " observed, %	5.78	6.05	5.53	5.75
$[\alpha]_D^{25}$ (H_2O), degrees	−52 (*c* 7.06)	−65 (*c* 3.61)	−59 (*c* 7.1)	
No. of titratable groups, acid	1	1	1	
" " " " basic	1	1	1	
pK′ of basic group	8.2	8.1	8.2	
Glucose, theoretical, equiv. per mole	1.00	1.00	1.00	1.00
Determined by anthrone	0.19	0.22	0.23	0.20
" " carbazole	0.04	0.06	0.06	
" " hot reduction of ferricyanide	0.99	1.05	0.93	0.99
Reduction, % of that by equivalent amount of synthetic fructose-glycine	122	100	140	116
Glucosamine color, % of that given by equivalent weight of glucosamine	10	9	9	10
Paper chromatography				
Butanol-acetic acid-water, R_F	0.14	0.087	0.087, 0.14	0.087, 0.14
Propanol-ammonia, R_F	0.21	0.16	0.15, 0.20	0.15, 0.20

the lower R_F, was ninhydrin-positive. On paper electrophoresis at pH 7 only one spot was obtained in each case. It may be that the relation of the material giving the two reducing spots with cold reagents is analogous to that of glutamic and pyrrolidonecarboxylic acids. If that is the case here, the compound with the higher R_F, which does not react positively with ninhydrin, would be the ring structure involving the hydrogen on the conjugated nitrogen.

Comparison of Band III Material with Synthetic Fructose-L-Alanine and Fructose-Glycine—Table II shows that the material in band III from both

dried and fresh hog liver was a mixture of fructose-L-alanine and fructose-glycine.

Comparison of Band I Material with Synthetic Fructose-L-Alanine and Fructose-Glycine (Table III)—Bands II and III behaved in every respect as the corresponding synthetic compounds or a mixture of them. Band I

TABLE III

Chemical and Physical Properties of Material Obtained from Band I Isolated from Dried Hog Liver

All values are calculated on the basis of 2 hexose and 1 amino acid residues per equivalent.

		Amino acid released on acid hydrolysis
		Alanine, glycine, traces of aspartic acid
Total nitrogen, %	Calculated	3.38–3.50
	Observed	3.67
No. of titratable groups	Acid	1
	Basic	1
Glucose, equiv. per mole		
Determined by anthrone	Calculated	1.2
	Observed	1.2
" " carbazole	Calculated	1.05
	Observed	0.9
" " hot reduction of ferricyanide	Calculated	1.00
	Observed	1.05
Reduction, % of that by equivalent amount of synthetic fructose-glycine	Calculated	112
	Observed	128
Glucosamine color, % of that given by equivalent weight of glucosamine	Calculated	10
	Observed	9.5
Paper chromatography		
Butanol-acetic acid-water, R_F		0.04
Propanol-ammonia, R_F		0.09

was different; it was eluted before fructose-glutamic acid; yet on acid hydrolysis the amino acids liberated were alanine and glycine, with a trace of aspartic acid. The filter paper chromatograms, unlike those of band III, gave only one spot with the ninhydrin or cold reduction reagents. An interpretation consistent with all the analytical data is that band I is a mixture of two compounds, each of which consists of two hexoses and one amino acid, either alanine or glycine. 1 of the 2 hexose residues is in the fructose-amino acid form, which would give low values with the anthrone and carba-

zole reagents and a nearly theoretical value in the hot reduction of ferricyanide. The other hexose residue is so linked that its power to reduce ferricyanide (in the hot or cold) is lost, but yields nearly the same value that free glucose does with the anthrone and carbazole reagents; this behavior is in accord with a normal glycosidic linkage. The values for reduction at room temperature and glucosamine color also are in accord with a structure consisting of two hexoses, one in a fructose-amino acid linkage

TABLE IV

Comparison of Alanine and Glycine Conjugates with Glucose, Mannose, or Galactose, with Band III Material from Dried or Fresh Hog Liver

Compound	F-Ala		F-Gly		T-Ala	T-Gly	Band III from	
Original sugar	Glucose	Mannose	Glucose	Mannose	Galactose	Galactose	Dried liver	Fresh liver
$[\alpha]_D^{25}$, degrees	−52	−55	−65	−66	−10.3	−13.5	−59	
c in H_2O	7.06	6.71	3.61	3.66	6.3	6.68	7.1	
Glucose, theoretical	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Determined by anthrone	0.19	0.19	0.23	0.22	0.12	0.17	0.23	0.20
Determined by carbazole	0.06	0.04	0.06	0.06	0.06	0.10	0.06	
Determined by hot reduction of ferricyanide	1.05	0.99	0.99	1.01	0.80	0.78	0.99	
Reduction, % of that by equivalent amount of synthetic fructose-glycine	124	124	100	104	224	208	140	116
Glucosamine color, % of that given by equivalent weight of glucosamine	10	10	10	9	10	10	9	10

and the other glycosidic. There was too little movement on the paper chromatograms to decide whether the material is a mixture of two such compounds, in one of which the amino acid is alanine and in the other glycine, or whether it is one compound consisting of four hexoses and two amino acids.

The hexose residue was identified by an indirect method, because treatment such as heating with acid, although it yields the constituent amino acid in low amount, does not give the sugar but furfural derivatives. The evidence in Table IV indicates that the hexose residue in the material of band III, obtained from dried or fresh liver, corresponds to fructose. In

the synthesis, a fructose-amino acid is obtained whether one uses glucose or mannose. In the condensation with the amino acid at the C-1 position of the sugar and the subsequent rearrangement the difference between glucose and mannose, which is at the C-2 position, is abolished. On the other hand the analogous compounds derived from galactose are tagatose-amino acids. The decisive comparison in determining the identity of the sugar radical is based on optical rotation. Table IV shows that F-Ala is the same, whether made from glucose or mannose, and that this is the case also with F-Gly, and band III corresponds to a mixture of F-Ala and F-Gly, and that these compounds are quite different from the analogous compounds made from galactose. The anthrone and carbazole sugar values were the same for all. Galactose was 70 per cent of the value given by glucose in the hot reduction of ferricyanide; correspondingly F-Ala, F-Gly (from either

TABLE V

Partial Reconstitution of Stimulation of Leucine Incorporation by Known Constituents of Liver Extract

Results expressed as per cent of blank control.

Added to control reaction mixture	None	Liver extract	F-Ala	F-Glu	F-Gly	Band I	Band II	Band III
None	100	170	100	100	100	100	100	100
Amino acids	160	350	160	160	160	160	160	160
" " + 0.5 γ Fe per ml.	210	350	290	260	285	290	290	290
" " + 5.0 " " " "	290	350	300	300	300	300	300	300

glucose or mannose), and band III gave nearly theoretical values by hot reduction of ferricyanide; T-Ala and T-Gly were lower. On the other hand reduction values for T-Ala and T-Gly were higher at room temperature than those for F-Ala, or F-Gly, which were the same whether the original sugar was glucose or mannose.

Stimulation of Amino Acid Incorporation—Table V presents the salient characteristics of the stimulation of the incorporation of labeled leucine into rabbit reticulocyte proteins by liver extract and by fructose-amino acids, whether synthetic or isolated from liver. Similar results were obtained with the incorporation of labeled glycine, histidine, and lysine. The liver extract referred to was prepared as described previously (1); 4 ml. of reaction mixture contained an amount equivalent to 0.5 gm. of fresh liver, which was optimal in the test system used. The results reported for the individual sugar-amino acids and for the material isolated from liver were those obtained with 5×10^{-4} M, which in every case was close to the lowest concentration giving maximal stimulation. The same results were obtained for bands I, II, and III from chromatograms of dried and fresh hog liver.

As Table V shows, the addition of the amino acid mixture plus iron nearly triples the rate of incorporation. Iron is ineffective unless the amino acids are added also; the amino acids stimulate alone, though less than the combination. The effect of the liver extract is doubled by the amino acids, but adding iron has no further augmenting effect, because the liver extract is rich in available iron. After removal of all heavy metals by 8-hydroxyquinoline, the liver extract had no stimulating effect; this effect was completely restored by the addition to the reaction mixture of 5 γ per ml. of ferrous iron. This concentration is the minimum for maximal stimulation, whether alone or with other stimulating factors. Ferric iron is only slightly less effective than ferrous iron.

Fructose-amino acids had no stimulating effect unless both the amino acid mixture and iron were added also. The concentration of added iron needs to be relatively low, *e.g.* 0.5 γ per ml. With 5.0 γ of iron the stimulation by iron plus amino acids is so high that added fructose-amino acids have only a slight effect. It appears that one of the functions of the fructose-amino acids is to make low concentrations of iron more effective.

DISCUSSION

The fructose-amino acids, whose isolation from liver and identification are described above, are the first to be found free in biological material. We are of the opinion that liver contains other fructose- (hexose-)amino acids in addition to these, indicated in the bands beyond III in Figs. 1 and 2. A number of synthetic fructose-amino acids were eluted from Dowex 50 columns in the same relative positions and required for elution the same strengths of trichloroacetic acid.

One point can be made at the present time regarding the mechanism of the stimulation of amino acid incorporation by fructose-amino acids. It is that a fructose-amino acid does not act as a carrier of its amino acid into the protein. There was no incorporation of leucine when fructose-C^{14}-L-leucine was used instead of C^{14}-L-leucine. The details of these experiments will be reported in a later communication, where the interrelation of the several stimulating factors we have found will be dealt with.

Even the greatest stimulation attainable by amino acids and iron, with or without the fructose-amino acid, falls short of the maximum obtained with amino acids plus liver extract. Because the maximal stimulation obtained with one fructose-amino acid compound was not augmented by the addition of ten other fructose-amino acids, our present, tentative interpretation is that there is another stimulating factor in liver.

The stimulating factor in plasma also requires the amino acids and iron; it differs from the fructose-amino acids we have so far isolated and synthesized in that its greatest effect is seen with a high iron concentration that obscures the stimulation by the fructose-amino acids. We are now engaged

in the isolation of this factor and the, as yet, unidentified factor in liver, which may be the same as the stimulating factor in plasma.

SUMMARY

1. A method is described of isolating fructose-amino acids (1-deoxy-1-amino-2-ketohexoses) from liver.

2. A test is described by which 10 γ of such compounds can be measured.

3. Aqueous extracts of dried and fresh liver contain a number of fructose-amino acids. Fructose-L-glutamic acid and a mixture of fructose-L-alanine and fructose-glycine were isolated and their identity proved by comparison with the synthetic compounds.

4. Some of the physical, chemical, and biological properties of the fructose-amino acids isolated are described.

5. Fructose-amino acids acting with certain amino acids and iron account for most of the stimulation exerted by the original liver extract on the incorporation of amino acids *in vitro* into the proteins of rabbit reticulocytes.

BIBLIOGRAPHY

1. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **196,** 669 (1952).
2. Kuhn, R., and Dansi, A., *Ber. chem. Ges.*, **69,** 1745 (1936).
3. Kuhn, R., and Weygand, F., *Ber. chem. Ges.*, **70,** 769 (1937).
4. Kuhn, R., and Birkofer, L., *Ber. chem. Ges.*, **71,** 621 (1938).
5. Weygand, F., *Ber. chem. Ges.*, **73,** 1259, 1284 (1940).
6. Fischer, E., *Ber. chem. Ges.*, **19,** 1920 (1886).
7. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27,** 1824 (1933).
8. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **179,** 689 (1949).
9. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83,** 115 (1929).
10. Dische, Z., *Mikrochemie*, **8,** 4 (1930).
11. Seibert, F. B., *J. Biol. Chem.*, **133,** 593 (1940).
12. Morris, D. L., *Science*, **107,** 254 (1948).
13. Park, J. T., and Johnson, M. J., *J. Biol. Chem.*, **181,** 149 (1949).
14. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192,** 663 (1951).
15. Hodge, J. E., and Rist, C. E., *J. Am. Chem. Soc.*, **75,** 316 (1953).
16. Gottschalk, A., *Biochem. J.*, **52,** 455 (1952).

XUM

ENZYMATIC DECARBOXYLATION OF MALONIC ACID*

By OSAMU HAYAISHI†

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, October 29, 1954)

In earlier communications (1, 2) we reported the participation of ATP[1] and CoA in the enzymatic decarboxylation of malonic acid and proposed the following sequence of reactions to account for these observations.

(1) $$\text{Malonate} + \text{CoA} \xrightarrow{\text{ATP}} \text{malonylmono CoA}$$

(2) $$\text{Malonylmono CoA} \rightarrow \text{acetyl CoA} + CO_2$$

(3) $$\text{Acetyl CoA} + \text{malonate} \rightarrow \text{malonylmono CoA} + \text{acetate}$$

In addition to these reactions, the acetate-activating system (Reaction 4) was also found in the crude extract.

(4) $$\text{Acetate} + \text{CoA} \xrightarrow{\text{ATP}} \text{acetyl CoA}$$

The present paper contains a full account of the study concerning the mechanism of malonate decarboxylation, carried out with a partially purified enzyme preparation obtained from malonate-adapted cells of *Pseudomonas fluorescens*.

Materials—Malonic acid disodium salt was a product of the Eastman Kodak Company. Monoethyl ester of malonic acid was prepared by mild alkaline hydrolysis of diethylmalonic acid (Eastman) at room temperature (3). Methylmalonic acid was prepared by methylation of diethylmalonate, followed by alkaline hydrolysis (4). Ethylmalonate was prepared by hydrolysis of diethylethylmalonate (Eastman). Ketomalonic acid was a product of the H. M. Chemical Company.

Carboxyl-labeled malonic acid was obtained by alkaline hydrolysis of 1,3-C^{14}-diethylmalonic acid (Nuclear Instrument and Chemical Corpora-

* This investigation was supported in part by a research grant (G3727) from the National Institutes of Health, United States Department of Health, Education, and Welfare.

† With the technical assistance of Natalie Ann Fraser and Takiko Hayaishi. Present address, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

[1] The following abbreviations will be used throughout the paper: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, 5′-adenylic acid; ITP, inosine triphosphate; CoA, coenzyme A; PTA, phosphotransacetylase; Tris, tris(hydroxymethyl)aminomethane.

tion). C^{14}-$BaCO_3$ and 1-C^{14}-sodium acetate were purchased from Tracerlab, Inc. ATP and CoA were products of the Pabst Laboratories. ADP, AMP, and Ba ITP were products of the Sigma Chemical Company, and Ba ITP was kindly furnished by Mr. K. Kurahashi of Western Reserve University after purification by ion exchange chromatography. Acetyl phosphate was a product of the Schwarz Laboratories, Inc. Hydroxamic acid derivatives of malonicmono- and diesters were prepared according to Hestrin (5). Purified phosphotransacetylase (6) was kindly furnished by Dr. Arthur Kornberg.

Determinations—Hydroxamic acid was determined by the method of Lipmann and Tuttle (7), except that acetate buffer was replaced by water. The hydroxamic acids were chromatographed and determined quantitatively, according to Stadtman and Barker (8), on Whatman No. 3 filter paper, except that acetone instead of ethanol was used for extraction.[2]

CO_2 was measured by a conventional Warburg technique in an atmosphere of pure nitrogen at 30°. Radioactivity was determined by direct plating of the material on aluminum disks after the pH was adjusted to about 10.0 with thymol blue as an indicator. Protein was determined by the method of Lowry *et al.* (9). Paper chromatography of malonic and acetic acids was carried out according to Isherwood and Hanes (10).

Preparation and Assay of Enzyme Fractions—Pseudomonas fluorescens strain 23 (11), a strictly aerobic microorganism, was grown approximately 20 hours at 30° with constant mechanical shaking in a medium containing 1 per cent NH_4Cl, 0.5 per cent disodium malonate, 0.15 per cent K_2HPO_4, 0.05 per cent KH_2PO_4, 0.02 per cent $MgSO_4 \cdot 7H_2O$, and 0.1 per cent Difco yeast extract.

Cell-free extracts were prepared by the method of McIlwain (12) with alumina (Alcoa A-301) as described before (11), except that grinding was carried out in the presence of reduced glutathione (1.0 mg. of the sodium salt per gm. of wet cells). Extraction was made with six parts of 0.02 M phosphate buffer (pH 7.0), and the mixture was centrifuged at 25,000 $\times g$ for 30 minutes (crude extracts).

To 50 ml. of crude extracts were added 50 ml. of 0.02 M phosphate buffer (pH 7.0) and 21 gm. of powdered ammonium sulfate. After the precipitate was discarded, 7 gm. of ammonium sulfate were added to the supernatant fluid. The resulting precipitate, collected by centrifugation, was dissolved in 15 ml. of 0.02 M phosphate buffer (pH 7.0) (Fraction I).

Crude extracts usually contain approximately 16 mg. of protein per ml. In an incubation mixture (2.0 ml.) containing 10 μmoles of glutathione, 100 μmoles of Tris buffer, pH 7.0, 100 μmoles of malonate, 1 μmole of $MgCl_2$, 0.02 μmole of CoA, and 1 μmole of ATP, about 2.8 μmoles of CO_2

[2] Suggested by Dr. I. Lieberman.

XUM

were liberated per mg. of enzyme protein at 30° during 10 minutes. Fraction I contains approximately 13 mg. of protein per ml., and about 25 μmoles of CO_2 were liberated per mg. of enzyme protein during a 10 minute period under comparable conditions.

Results

Cofactor Requirement—Marked stimulation of the rate of enzymatic decarboxylation of malonate in the presence of ATP and CoA was demonstrated either by treatment of crude extracts with Dowex 1 and charcoal, as previously reported (1), or by using partially purified enzyme prepara-

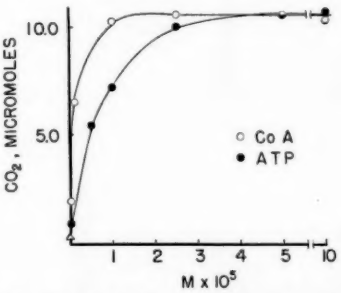


FIG. 1. Influence of ATP and CoA on the rate of malonate decarboxylation. The incubation mixture (2.0 ml.) contained 0.1 ml. of Fraction I, 10 μmoles of glutathione, 100 μmoles of KF, 40 μmoles of Tris buffer, pH 7.2, 50 μmoles of malonate, 1 μmole of $MgCl_2$, ATP as indicated (in the presence of 0.02 μmole of CoA), or CoA as indicated (in the presence of 0.1 μmole of ATP). Incubation time, 5 minutes at 30°, after which 100 μmoles of H_2SO_4 were tipped in. △ = CO_2 evolution when both CoA and ATP were omitted.

tions (Fig. 1). Practically no CO_2 evolution was observed when both ATP and CoA were omitted from the reaction mixture. Potassium fluoride was added to inhibit ATPase, and $MgCl_2$ was routinely added to the incubation mixture (see below). The affinity of ATP and CoA for the enzyme was relatively high, and maximal velocity was attained with approximately 10^{-5} M CoA and 5×10^{-5} M ATP. It was also noteworthy that only catalytic amounts of ATP and CoA were required for an extensive decarboxylation of the substrate.

ADP was about 25 per cent as active as ATP under the test conditions when ADP was incubated in the main compartment of the Warburg vessels with the enzyme. However, the effect of ADP was only negligible when it was tipped into the main compartment at the same time the substrate was. Further studies revealed the presence of adenylkinase in Fraction I. This activity was almost completely lost by boiling in 0.05 N HCl for 10 minutes

and, therefore, differs from conventional muscle myokinase in this respect, but resembles the liver myokinase described by Kotelnikova (13). AMP was completely inactive, while ITP was almost as active as ATP.

Metal Requirement—Versene (ethylenediaminetetraacetic acid) (0.5×10^{-2} M) did not affect the rate of the decarboxylation reaction under the standard assay conditions with Fraction I as an enzyme source. However, when Fraction I was dialyzed against running distilled water at 3° for 6

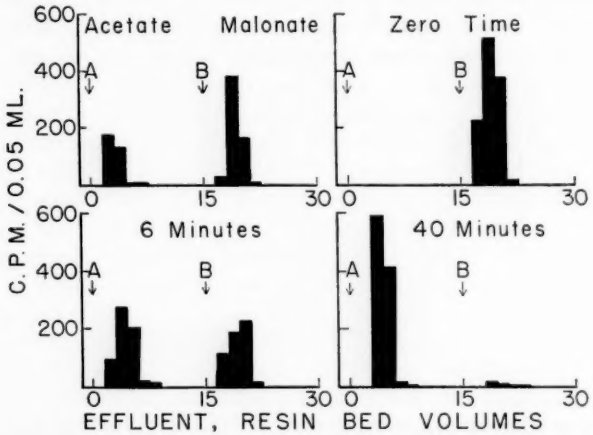


FIG. 2. Stoichiometry of malonate decarboxylation. The conditions are essentially the same as those described for Fig. 1, except that 40 μmoles of radioactive malonate containing 5.1×10^5 c.p.m. were used as substrate, 0.2 ml. of Fraction I was used, and H_2SO_4 was tipped in after 6 and 40 minutes as indicated. After deproteinization, 0.05 ml. aliquots were used for radioautograph (Fig. 3), and 0.5 ml. aliquots were chromatographed on a Dowex 1 formate column (1 sq. cm. × 5 cm.). Elution was carried out with 0.05 M HCl (*A*); after 15 resin bed volumes, this was replaced with 0.1 M HCl (*B*).

hours, about 50 per cent of the activity was lost. Approximately 20 per cent stimulation was observed with the remaining activity in the presence of 10^{-3} M Mg^{++}. Mn^{++} (10^{-3} M) showed only a slight stimulation, while Co^{++} and Zn^{++} (10^{-3} M) were slightly inhibitory.

Stoichiometry—Balance studies of the over-all reaction were made with 1,3-C^{14}-labeled malonic acid as a substrate. The rate of the decarboxylation was determined by manometry, and, after the reaction was over, acid was tipped in from the side arm and the total CO_2 released was determined. The contents were centrifuged to remove the protein precipitate, a small amount of carrier malonate and acetate was added, and an aliquot from the supernatant fluid was subjected to Dowex 1 chromatography and to

paper chromatography as well. As shown in Fig. 2, authentic samples of acetic acid and malonic acid can be separated by differential elution and determined quantitatively by the measurement of radioactivity. In one case, the reaction was stopped after 6 minutes when it was about 50 per cent over, as judged by CO_2 evolution. With 40 μmoles of malonate, 21.3 μmoles of CO_2 were evolved. 20.4 μmoles of malonate disappeared and 18.4 μmoles of acetate appeared. In another case the sample was incubated 40 minutes, at which time CO_2 evolution was essentially complete.

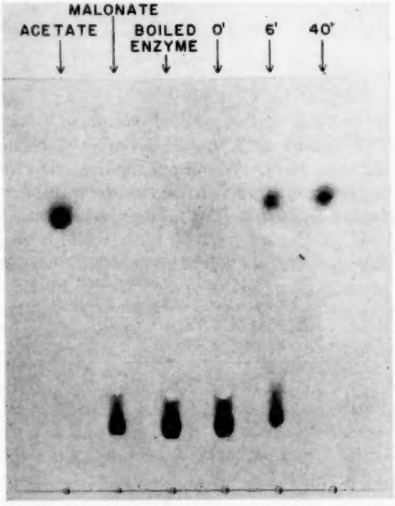


FIG. 3. Radioautogram of the incubation mixture, malonate, and acetate. Conditions as described in the legend to Fig. 2. 0.05 ml. aliquots containing about 13,000 c.p.m. were put on Whatman No. 3 filter paper, developed with *n*-propanol ammonia mixture (60:40), and the radioautogram made by exposure for 4 days.

39.2 μmoles of CO_2 were evolved, 39.4 μmoles of malonate disappeared, and 36.4 μmoles of acetate appeared. In both experiments, acetate was the only product which accumulated in a detectable amount.

Since the recovery of a known amount of malonate and acetate under these conditions varied between 85 and 100 per cent, the results seem to indicate that the over-all reaction is a stoichiometric decarboxylation of malonic acid to yield acetic acid and CO_2. Similar findings were obtained by the radioautogram (Fig. 3). Again the appearance of acetate was matched by the disappearance of malonate, and no other intermediate compounds or products were found to accumulate to any detectable extent. When the spots were cut out and the radioactive material was eluted and

counted, essentially similar results as before were obtained, indicating that the over-all reaction is a decarboxylation of malonate to yield acetate and CO_2.

Trapping Experiments with Hydroxylamine—When hydroxylamine was added to the reaction mixture, derivatives of hydroxamic acid were produced and CO_2 evolution was strongly suppressed. The results with different levels of hydroxylamine are reported in Table I. The hydroxamic acid derivatives were identified from the R_F values on paper as acethydroxamic acid (R_F = 0.51 to 0.54) and malonmonohydroxamic acid (R_F = 0.35 to 0.38). As the concentration of hydroxylamine was increased, the production of the total hydroxamate and acethydroxamate was decreased, indicating that hydroxylamine may be inhibitory to the enzyme. However, at higher levels of hydroxylamine, more malonmonohydroxamate was trapped. These observations indicated that the primary product was probably malonylmono CoA and that hydroxylamine was competing with decarboxylase for this compound. In other words, at higher levels of hydroxylamine, more malonylmono CoA was trapped, and therefore decarboxylation was greatly suppressed and a relatively small amount of acethydroxamic acid was formed, whereas, at the lower concentration of hydroxylamine, more malonyl CoA was split into CO_2 and acetyl CoA, the latter being converted either directly to acethydroxamate or enzymatically into free acetate, which may be activated again to yield acetyl CoA. Nei-

TABLE I

Effect of Different Concentrations of Hydroxylamine on Hydroxamate Formation

The incubation mixture (2.0 ml.) contained 0.6 ml. of Fraction I, 100 μmoles of KF, 100 μmoles of ATP, 0.2 μmole of CoA, 5 μmoles of glutathione, 2 μmoles of $MgCl_2$, 100 μmoles of malonate, and hydroxylamine as indicated. Incubation, 1 hour at 30°. 1 ml. aliquot was used for total hydroxamate determination and 1 ml. aliquot for paper chromatographic analysis. The values are expressed as micromoles per 2.0 ml.

	Amount of hydroxylamine					
	1600 μmoles	1200 μmoles	800 μmoles	400 μmoles	1200 μmoles*	1200 μmoles†
Total hydroxamate	1.60	2.90	5.25	6.10	0.06	0.15
Acethydroxamate	0.88	1.85	4.50	5.00		
Malonmonohydroxamate	0.45	0.25	Trace‡	0.0		

* With boiled enzyme preparation.

† Without malonate.

‡ Only detectable on the paper.

ther malondihydroxamic acid ($R_F = 0.1 \sim 0.15$) nor formhydroxamic acid ($R_F = 0.40 \sim 0.44$) was detected.

Separation of Acetate-Activating Enzyme and Malonate Decarboxylase—175 ml. of calcium phosphate gel (16.5 mg. per ml.) were centrifuged, 50 ml. of crude extract were added to the solid gel, and the mixture stirred about 20 minutes at 0°. After centrifugation, the supernatant fluid was treated with 50 ml. of the calcium phosphate gel in the same manner, and the supernatant solution containing about 1.5 mg. of protein per ml. was designated the gel supernatant fraction. The gel was eluted with 50 ml. of 0.02 M phosphate buffer (pH 7.0) and then with 20 ml. of 0.05 M phosphate buffer (pH 7.0). The gel supernatant fraction contained an acetate-

TABLE II

Separation of Malonate Decarboxylase and Acetate Activating Enzyme

For hydroxamate determination, the incubation mixture (1.0 ml.) contained 0.25 ml. of gel supernatant fraction (Gel. Sp.) or gel eluate (Gel. El.), 50 μmoles of malonate or acetate, 500 μmoles of KF, 50 μmoles of ATP, 10 μmoles of glutathione, 0.1 μmole of CoA, 5 μmoles of $MgCl_2$, and 1000 μmoles of NH_2OH. Incubation time, 30 minutes. For CO_2 determination, the conditions were the same, except that NH_2OH was omitted and 100 μmoles of Tris buffer, pH 7.0, were added. Incubation time, 10 minutes.

Substrate	Product	Enzyme preparation		
		Gel. Sp.	Gel. El.	Gel. Sp. + Gel. El.
		μmoles	*μmoles*	*μmoles*
Malonate	Hydroxamic acid derivative	0.07	0.19	1.79
Acetate	" " "	1.41	0.17	1.75
Malonate	CO_2	0.0	11.0	12.0

activating system similar to that of yeast or animal tissues and was completely devoid of a malonate decarboxylase system. The gel eluate, on the other hand, contained a malonate decarboxylase, but was practically free of an acetate-activating enzyme (Table II). When malonate was used as a substrate, neither fraction alone formed an appreciable quantity of the hydroxamic acid derivatives. However, when they were combined, the amount of hydroxamic acid produced was increased nearly 10-fold. When chromatographed on paper, about 90 per cent of the hydroxamic acid derivatives was found to be acethydroxamic acid, the rest being malonmonohydroxamic acid. These results also indicate that free acetate is probably the direct product of malonate decarboxylation and that with the crude extract most of the acethydroxamate was produced by a secondary reaction according to Reaction 4.

Rôle of Acetyl CoA—In the presence of PTA and CoA, acetyl phosphate

could replace ATP, indicating that acetyl CoA is an integral part of the reaction mechanism involving malonate decarboxylation. Since crude extracts do not contain PTA, the addition of acetyl phosphate alone did not stimulate decarboxylation, even in the presence of CoA. Nor did the addition of purified PTA affect the rate of the reaction in the absence of acetyl phosphate. However, when both were added together, the rate of decarboxylation was increased more than 10-fold, indicating that acetyl CoA

TABLE III

Effect of Acetyl Phosphate and Phosphotransacetylase

The incubation mixture (2.0 ml.) contained 50 μmoles of malonic acid, 0.06 ml. of Fraction I, 20 μmoles of glutathione, 100 μmoles of Tris buffer (pH 7.4), 2 μmoles of $MgCl_2$, and 0.05 μmole of CoA. Incubation time, 10 minutes.

Addition	CO_2
	μmoles
None	0.9
Acetyl phosphate (5 μmoles)	1.0
PTA (21 units)	0.4
Acetyl phosphate + PTA	9.8

TABLE IV

Effect of Arsenate and Phosphotransacetylase

Conditions as for Table III.

Addition	CO_2	
	+ 10 μmoles ATP	+ 1 μmole ATP
	μmoles	*μmoles*
None	10.4	11.8
Arsenate (20 μmoles)	8.6	9.4
PTA (4 units)	9.7	11.7
Arsenate + PTA	4.6	1.2

can replace ATP and CoA in this system (Table III). Conversely, in the presence of ATP and CoA, neither arsenate nor transacetylase alone affected the rate of the reaction more than 20 per cent, but, when both were combined, the degree of inhibition was considerably increased, since acetyl CoA was arsenolyzed in the presence of arsenate, CoA, and PTA and was no longer available for the CoA transferase reaction (Reaction 3). This effect was even more pronounced when the level of ATP was relatively low (Table IV), because, in the absence of Reaction 3, the rate and the extent of Reaction 2 may become dependent upon Reaction 1, the latter being determined by the amount of ATP present in the reaction mixture.

Influence of pH and Substrate Concentration—The pH optimum and the substrate affinity of the over-all reaction were determined with Fraction I by manometric technique. Maximal activity for the decarboxylation was observed in the range of pH 6.5 ~ 7.0 (Fig. 4).

The influence of varying the substrate concentration is illustrated in Fig. 5. The apparent dissociation constant (K_m) of the enzyme and substrate complex is approximately 2×10^{-3} M.

Substrate Specificity—Malonic acid was the only substrate which was found to be decarboxylated by Fraction I. Keto-, methyl, and ethyl malonate were all inactive. Likewise oxalacetic acid, oxalic acid, glutaric

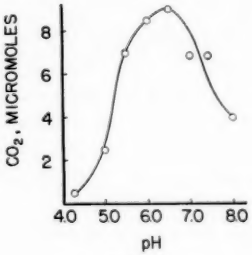


FIG. 4

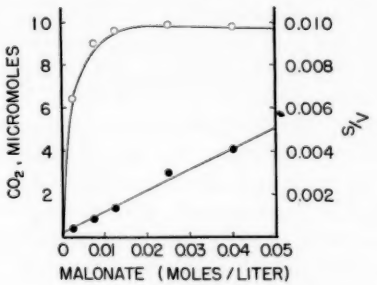


FIG. 5

FIG. 4. Rate of decarboxylation as a function of pH. Conditions as described for Fig. 1, except that acetate buffer was used for pH 4.2, 5.0, and 5.6, and phosphate buffer for pH 6.0, 6.5, and 7.0.

FIG. 5. Rate of decarboxylation as a function of substrate concentration. Conditions as described for Fig. 1. The right-hand ordinate scale refers to a Lineweaver-Burk plot of substrate concentration divided by velocity (S/V) as a function of substrate concentration.

acid, and succinic acid were not decarboxylated. The rigid specificity of this enzyme makes it an excellent tool for specific and quantitative determination of malonic acid. The crude extract catalyzed the formation of the hydroxamic acid derivatives from succinate, propionate, acetate, and malonate, but not from other fatty acids or dicarboxylic acids. However, when the extracts were made from cells grown on succinate, only succinate, propionate, and acetate were activated. Extracts from cells grown on a glucose medium did not show any of these activities.

Irreversibility of Malonate Decarboxylation—50 μmoles of $NaHCO_3$ containing 5×10^5 c.p.m. were incubated in a total volume of 1.0 ml. with 50 μmoles of acetate in the presence of 200 μmoles of Tris buffer, pH 7.0, 10 μmoles of glutathione, 0.05 μmole of CoA, 5 μmoles of ATP, 2 μmoles of $MgCl_2$, and 0.5 ml. of Fraction I, for 1 hour at 30°. The incubation mixture was chilled and deproteinized by the addition of 0.1 ml. of 5 N H_2SO_4

in the cold, and 50 μmoles of potassium malonate were added as carrier. When CO_2 was bubbled through the supernatant solution, essentially no radioactivity remained in the reaction mixture. Similar experiments with radioactive acetate also failed to show any net synthesis of malonate. Since the free energy change of malonate → acetate + CO_2 is calculated to be roughly −7000 calories per mole (14), it is not unreasonable to expect that, with this amount of radioactivity, synthesis of malonate from acetate and CO_2 could not be demonstrated.

Distribution of Malonate Decarboxylase—Since malonic acid has been reported to be utilized by microorganisms (15–19) and certain tissues of mice (20) and rats (21), as well as by fish muscle (22), the distribution of malonate decarboxylase was investigated in the presence and absence of ATP and CoA, with labeled malonate as a substrate, in order to pick up any relatively weak activity in the various organs of mammals and fishes. In confirmation of the previous report by Lifson and his coworkers (20, 21), kidney, and liver to a lesser extent, seems to have some decarboxylase activity. However, the specific activity of the crude kidney homogenate of the rat is approximately 0.0001 that of the crude extracts of malonate-adapted *Pseudomonas*. We were not able to observe any activity in homogenates of the spleen, brain, and skeletal muscle of the rat, nor with the dorsal muscle and liver of the carp, nor with the muscle of catfish, lobster, or shrimp.

DISCUSSION

In 1946, Vennesland *et al.* (23) reported that malonic acid is produced from oxalacetic acid in the presence of Mn^{++} and a crystalline metmyoglobin preparation from horse liver. A similar observation was recently made with plant extracts (24). Malonic acid was also shown to be a product of uracil degradation by bacterial enzymes (25–27). Although the utilization of malonic acid by microorganisms (15–19), as well as by mammals (20, 21) and fishes (22), has been reported, its precise metabolic pathways have not been elucidated to date. Recently Wolfe, Ivler, and Rittenberg (28, 29) confirmed and extended our preliminary results and have proposed a mechanism of malonate decarboxylation whereby dimalonyl CoA plays a key rôle in the CoA transferase couple.

The data presented in this paper fail to offer evidence for the presence of dimalonyl CoA, although the possibility of its existence is not excluded. The available evidence suggests that in the primary reaction monomalonyl CoA is produced from malonic acid in the presence of ATP and CoA. Although the detailed mechanism of this reaction is not yet clear, malonyl phosphate does not seem to be involved as an intermediate in this reaction, since the incubation of malonate and the enzyme with ATP alone did not

lead to formation of hydroxamic acid derivatives. Once a catalytic amount of malonyl CoA was produced, it was decarboxylated to yield acetyl CoA. Then CoA was transferred from acetyl CoA to another molecule of malonic acid, and free acetate was liberated, yielding malonyl CoA by a cyclic mechanism, as shown in the accompanying scheme (Fig. 6).

The transfer of CoA from one fatty acid to another has been described by Stadtman (30) and more recently by Lieberman (31). Beinert and Stansly (32) have recently reported malonate as a most favorable acceptor in the CoA transferase system from the pig heart.

The rate of direct activation of malonate by CoA and ATP (Reaction 1) appears to be much slower than the CoA transferase reaction (Reaction 3), since, when a small amount of enzyme was used, a lag in CO_2 evolution was observed which could be abolished by adding acetate to the reaction mixture and preincubating with ATP and CoA to generate acetyl CoA. Furthermore, only a catalytic amount of ATP was utilized for extensive decarboxylation of malonate, even if excess ATP was present in the incubation mixture.

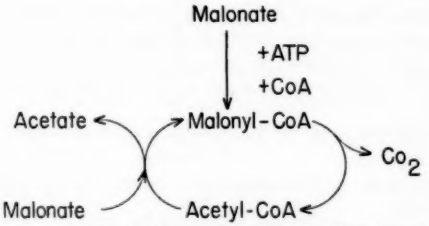


FIG. 6. A proposed scheme of malonate decarboxylation

These results, together with the studies on succinate decarboxylation by Whiteley (33, 34) and Delwiche *et al.* (35), seem to indicate that this type of decarboxylation is commonly employed by both aerobic and anaerobic microorganisms in their metabolism of the dicarboxylic acids. Although weak malonate decarboxylase activity was observed in mammals, it has not been determined whether it requires ATP and CoA, because the rate of the reaction was too slow.

SUMMARY

The mechanism of enzymatic decarboxylation of malonic acid was studied with a partially purified preparation obtained from malonate-adapted cells of a *Pseudomonas* species. Available evidence indicates the following sequence of reactions as a plausible mechanism. In the primary reaction malonylmono CoA is produced from malonate and CoA in the

presence of ATP. Malonylmono CoA is then decarboxylated to produce acetyl CoA. Once a catalytic amount of acetyl CoA is produced, CoA is transferred from acetyl CoA to another molecule of malonate by the action of CoA transferase. Thus acetate is produced as a product yielding malonyl CoA by a cyclic mechanism.

BIBLIOGRAPHY

1. Hayaishi, O., *J. Am. Chem. Soc.*, **75,** 4367 (1953).
2. Hayaishi, O., *Federation Proc.*, **13,** 226 (1954).
3. Freund, M., *Ber. chem. Ges.*, **17,** 780 (1884).
4. *Org. Syntheses*, 2nd edition, coll. **1,** 250, New York (1941).
5. Hestrin, S., *J. Biol. Chem.*, **180,** 249 (1949).
6. Stadtman, E. R., *J. Biol. Chem.*, **196,** 535 (1952).
7. Lipmann, F., and Tuttle, L. C., *Biochim. et biophys. acta*, **4,** 301 (1950).
8. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184,** 769 (1950).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
10. Isherwood, F. A., and Hanes, C. S., *Biochem. J.*, **55,** 824 (1953).
11. Hayaishi, O., and Stanier, R. Y., *J. Bact.*, **62,** 691 (1951).
12. McIlwain, H., *J. Gen. Microbiol.*, **2,** 288 (1948).
13. Kotelnikova, A. V., *Doklady Akad. Nauk S. S. S. R.*, **59,** 527 (1948); *Chem. Abstr.*, **42,** 6388 (1948).
14. Parks, G. S., and Huffman, H. M., Free energies of some organic compounds, New York (1932).
15. den Dooren de Jong, L. E., Bijdrage tot de kennis van het mineralisatieproces, Rotterdam (1926).
16. Karlsson, J. L., *J. Biol. Chem.*, **183,** 549 (1950).
17. Challenger, F., Subramaniam, V., and Walker, T. K., *J. Chem. Soc.*, 200 (1927).
18. Burris, R. H., and Wilson, P. W., *Cold Spring Harbor Symposia Quant. Biol.*, **7,** 349 (1939).
19. Gray, C. T., *J. Bact.*, **63,** 813 (1952).
20. Lifson, N., and Stolen, J. A., *Proc. Soc. Exp. Biol. and Med.*, **74,** 451 (1950).
21. Lee, J. S., and Lifson, N., *J. Biol. Chem.*, **193,** 253 (1951).
22. Yamada, K., and Suzuki, T., *J. Agr. Chem. Soc. Japan*, **25,** 290, 370 (1952).
23. Vennesland, B., Evans, E. A., Jr., and Francis, A. M., *J. Biol. Chem.*, **163,** 573 (1946).
24. Kenten, R. H., and Mann, P. J. G., *Biochem. J.*, **53,** 498 (1953).
25. Hayaishi, O., *Federation Proc.*, **11,** 227 (1952).
26. Lara, F. J. S., *J. Bact.*, **64,** 279 (1952).
27. Hayaishi, O., and Kornberg, A., *J. Biol. Chem.*, **197,** 717 (1952).
28. Wolfe, J. B., Ivler, D., and Rittenberg, S. C., *J. Biol. Chem.*, **209,** 867, 875 (1954).
29. Wolfe, J. B., and Rittenberg, S. C., *J. Biol. Chem.*, **209,** 885 (1954).
30. Stadtman, E. R., *J. Biol. Chem.*, **203,** 501 (1953).
31. Lieberman, I., *Arch. Biochem. and Biophys.*, **51,** 350 (1954).
32. Beinert, H., and Stansly, P. G., *J. Biol. Chem.*, **204,** 67 (1953).
33. Whiteley, H. R., *J. Am. Chem. Soc.*, **75,** 1518 (1953).
34. Whiteley, H. R., *Proc. Nat. Acad. Sc.*, **39,** 772, 779 (1953).
35. Delwiche, E. A., Phares, E. F., and Carson, S. F., *Federation Proc.*, **12,** 194 (1953).

DEGRADATION OF GLUCOSE-C^{14} WITH LEUCONOSTOC MESENTEROIDES; ALTERNATE PATHWAYS AND TRACER PATTERNS*

By I. A. BERNSTEIN,† KENNETH LENTZ, MIGNON MALM,‡ PER SCHAMBYE,§ and HARLAND G. WOOD

(*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio*)

(Received for publication, November 22, 1954)

The pattern of C^{14} in the glucose of liver glycogen has been used frequently as an indicator of metabolism, and uniformly the distribution has been found to be in accord with traditional schemes of glycolysis and the tricarboxylic acid cycle (1). Sufficient evidence is now available, however, to indicate that there are other pathways for the metabolism of glucose (2–7) which in certain microorganisms even represent major routes of metabolism (2–4). In animals the relative importance of these pathways has been studied, and it has been concluded that certain tissues and organs depend on glycolysis or the alternate pathways almost entirely, while others utilize a combination of both (8–10). In liver slices, for example, it is reported (8) that 75 per cent or more of the oxidation of glucose to CO_2 proceeds by pathways other than glycolysis.[1]

Fig. 1 represents the hexose monophosphate oxidative cycle which, together with glycolysis and the tricarboxylic acid cycle, possibly accounts for glucose metabolism in animals (5, 6, 12, 13). In the hexose mono-

* This investigation has been supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, by the Atomic Energy Commission under contract No. AT(30-1)-1320, and by the Elisabeth Severance Prentiss Fund, Western Reserve University. The C^{14} was obtained on allocation from the Atomic Energy Commission.

† Present address, Institute of Industrial Health, University Hospital, University of Michigan, Ann Arbor, Michigan.

‡ Fellow of the American Association of University Women. Present address, School of Veterinary Medicine, University of Stockholm, Stockholm, Sweden.

§ Fulbright Traveling Fellow. Present address, Department of Special Pathology and Therapy, The Royal Veterinary and Agricultural College, Copenhagen, Denmark.

[1] The method of calculation has been questioned by Katz *et al.* (11). They estimate that 79 to 84 per cent of the CO_2 which is derived from the carbon of the added glucose arises via glycolysis. Both calculations appear to be correct on the basis of the assumptions made. Bloom *et al.* compare the yield of CO_2 from *carbon 1* glycolytically and via the alternate pathway, whereas Katz *et al.* compare the yield from all 6 carbons glycolytically with that from *carbon 1* via the alternate pathway.

phosphate oxidative cycle, 3 molecules of hexose monophosphate are converted to 3 of CO_2, 1 of triose phosphate, and 2 of hexose monophosphate (5, 14, 15). If this pathway operates as shown in Fig. 1, then hexose monophosphate labeled in carbons 3 and 4 would yield hexose monophosphate-2,3,4-C^{14}, which, on further passage through the cycle, would result in label in carbon 1. Carbons 5 and 6 would remain free of tracer by this process. Horecker *et al.* (15) have tested this hypothesis, using C^{14}-labeled pentose phosphates, and have obtained results in accord with the scheme in Fig. 1. Therefore, if the cycle as diagramed were operating *in vivo*, the pattern of tracer in the hexose monophosphate should be unsymmetrical, provided randomization of the label by triosephosphate isomerase were incomplete.

Previous investigations (1) with $NaHC^{14}O_3$ in fasted rats have shown that the label is predominantly in positions 3 and 4 of the liver glycogen, although all the other carbons contained some activity. The principal purposes of the present study were to determine (*a*) the activities in carbons 1, 2, 5, and 6 and (*b*) whether the distribution of tracer was unsymmetrical and in agreement with the predictions of the hexose monophosphate oxidative cycle.

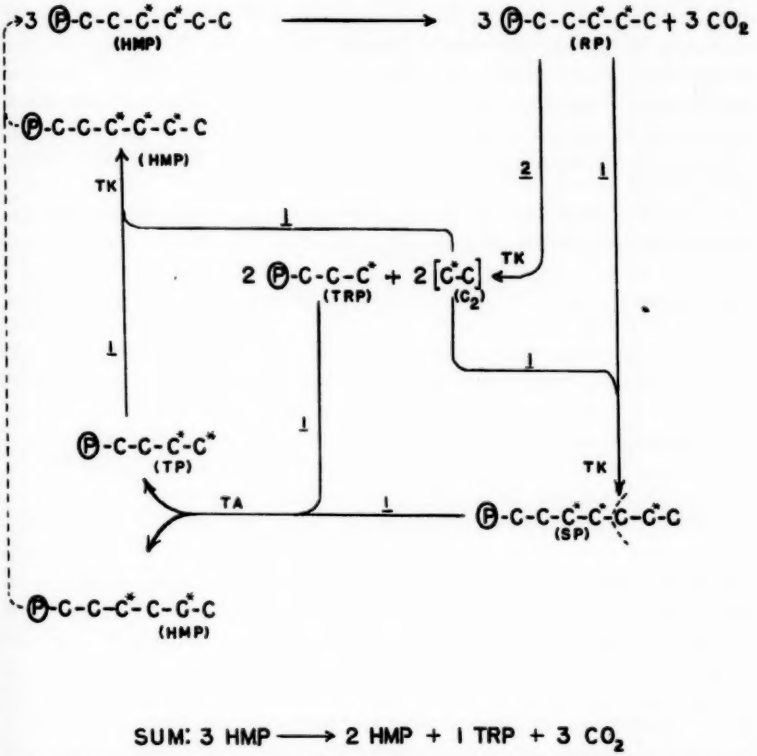
To approach this problem a convenient and rapid method was needed for determining the level of tracer in each carbon of glucose. Gunsalus and Gibbs (2) demonstrated the potential usefulness of the *Leuconostoc mesenteroides* fermentation for this purpose, but their data did not unequivocally demonstrate its reliability, since the C^{14} balances were not entirely satisfactory.[2] To investigate the validity of this method, four different types of labeled glucose have been degraded with *L. mesenteroides* and compared with degradations by *Lactobacillus casei* and by periodate oxidation of potassium gluconate and the glucobenzimidazole derivative. It is shown that glucose may be degraded with *L. mesenteroides*, yielding each carbon atom directly and with no significant cross-contamination of C^{14}. Degradation of highly active glucose from liver glycogen yielded no clear indication of a major rôle for the alternate pathway in glucose metabolism.

Methods

Source of Glucose-C^{14}—The glucose-3,4-C^{14} samples were obtained by hydrolyzing glycogen isolated from the livers of rats. The animals were fasted for 48 hours, given by stomach tube 200 mg. of glucose and by intra-

[2] Horecker *et al.* (15) used the *L. mesenteroides* fermentation to degrade glucose in their most recent investigation and confirmed their data with yeast and *Pseudomonas lindneri* fermentations, which yield the 3 and 4 carbons and the 1 and 4 carbons, respectively, as CO_2.

peritoneal injection 1.6 ml. of 0.25 M $NaHC^{14}O_3$ per 100 gm. of body weight, and sacrificed after 4 hours. The $NaHC^{14}O_3$ was administered in eight equal aliquots at zero time and every half hour thereafter. The activity



SUM: 3 HMP ⟶ 2 HMP + 1 TRP + 3 CO_2

FIG. 1. Hypothetical concept of the hexose monophosphate oxidative cycle in animal tissues. TRP = triose phosphate, HMP = hexose monophosphate, RP = ribose phosphate, C_2 = hypothetical 2 carbon unit, SP = sedoheptulose phosphate, TP = tetrose phosphate, TK = transketolase, TA = transaldolase; an asterisk denotes an isotopic tracer. The underlined numerals indicate the number of molecules traversing the particular pathway.

of the $NaHC^{14}O_3$ used was 0.8 mc. per mmole in Experiment 1 and 1.5 mc. per mmole in Experiment 2.

Glucose-C^{14} labeled in all positions (but not uniformly) was prepared from glycogen isolated from the combined internal organs of chicks fed $HC^{14}OONa$ (16). Glycogen was isolated as described by LePage (17) from

a trichloroacetic acid extract of the ground tissue and hydrolyzed in H_2SO_4 according to the method of Wood *et al.* (18). The sulfate was removed by neutralization to bromocresol purple with barium hydroxide, and the hydrolysate was chromatographed for 18 hours at room temperature on 50 cm. × 50 cm. sheets of Eaton-Dikeman No. 627-030 filter paper by the ascending technique (19) with a mixture by volume of 5 parts of ethyl acetate, 2 of pyridine, and 5 of water (20). The glucose band was located (21, 22) and eluted with water. Carrier glucose was added and the hexose crystallized by concentration to a syrup and seeding.[3]

Glucose-2-C^{14} and glucose-6-C^{14} were obtained from the National Bureau of Standards.

Degradation of Glucose by L. mesenteroides—The degradation was accomplished by the following procedures.

$$CH_2OH(CHOH)_4CHO \xrightarrow{L.\ mesenteroides} CH_3CHOHCOOH + HOH_2CCH_3 + CO_2$$
(C-1)

$$CH_3CH_2OH \xrightarrow{H_2Cr_2O_7} CH_3COOH$$

$$(CH_3COO)_2Ba \xrightarrow{\Delta} CH_3COCH_3 + BaCO_3 \quad (C\text{-}3) \qquad CH_3COCH_3 \xrightarrow{NaOI} CHI_3 \quad (C\text{-}2)$$

$$CH_3CHOHCOOH \xrightarrow{MnO_2} CH_3CHO + CO_2 \quad (C\text{-}4) \qquad CH_3CHO \xrightarrow{NaOI} CHI_3 \quad (C\text{-}6) + HCOOH \quad (C\text{-}5)$$

L. mesenteroides, strain 39, was maintained by monthly transfer in a stock agar.[4] Cells for fermentation were grown in a medium containing 1 per cent glucose, 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent dibasic potassium phosphate, 10 per cent (by volume) tomato juice, and 2 per cent by volume of salt mixture (containing 0.8 per cent magnesium sulfate, 0.04 per cent sodium chloride, 0.04 per cent ferric sulfate, 0.16 per cent manganese sulfate, and 0.02 per cent ascorbic acid). The salt and glucose solutions were sterilized separately. The remaining components were autoclaved for 20 minutes, cooled, the denatured protein was removed by filtration or centrifugation, and the supernatant solution was

[3] Addition of unlabeled glucose at this point resulted in a 5000-fold dilution of the labeled hexose in Experiment 1 and a 97-fold in Experiment 2. These sugars were degraded without further dilution.

[4] The media used were suggested by Dr. M. Gibbs in a personal communication. The stock agar contained 0.05 per cent glucose, 1 per cent tryptone, 0.25 per cent manganese phosphate, 0.02 per cent manganese sulfate, 0.4 per cent magnesium sulfate, 0.5 per cent agar, 0.3 per cent calcium carbonate, about 0.02 per cent thiamine, and 10 per cent (by volume) of beef liver extract (prepared by homogenizing 1 pound of liver in 2 liters of water in a Waring blendor for 2 minutes and filtering with suction through a thin Hyflo Super-Cel mat on Whatman No. 5 filter paper.

sterilized. 10 ml. of the medium were inoculated from a stab culture and incubated until evolution of gas was observed (about 24 hours). Successive 10 ml. subcultures were made (with 1 drop of inoculum) until evolution of gas occurred in 12 hours. Cultures were then successively transferred into 100 ml. and 1 liter of medium with a 1 per cent inoculum. Each culture was incubated until gas evolution was profuse (about 10 to 12 hours). All incubations were at 30°. The cells were harvested[5] by centrifugation, washed twice with distilled water, suspended in distilled water (2 gm. per 10 ml.), and used immediately.

The fermentations were carried out at 30° under nitrogen in 125 ml. Warburg vessels with final concentrations of 0.033 M glucose, 0.5 M potassium phosphate buffer at pH 6, and 2.5 per cent suspension of cells in a final volume of 30 ml. In addition 2 ml. of 2.5 M NaOH (carbonate-free) with filter paper wick were put in the center well for absorption of the evolved CO_2. The progress of the fermentation was followed manometrically in a small Warburg vessel without NaOH in the center well. When there was no further increase in pressure[6] in this flask, the alkali and filter paper in the large flask were removed for determination of the specific activity of the CO_2 (C-1). The cells were removed by centrifugation, and, after the solution was neutralized to phenol red with NaOH, the ethanol was recovered by distillation of three-fourths of the volume. The lactic acid was obtained by continuous ether extraction of the acidified residue and was degraded[7] by the method of Wood *et al.* (18) to CO_2 (C-4 of the glucose), HCOOH (C-5), and CHI_3 (C-6). The ethanol after redistillation from neutral solution as above was oxidized to acetic acid in a stoppered flask by adding 15 ml. of a potassium dichromate solution in H_2SO_4 (134 gm. of $K_2Cr_2O_7$ + 675 ml. of 10 N H_2SO_4 diluted to 1 liter) and heating in a boiling water bath for 30 minutes. The acetic acid was recovered by steam distillation (10 volumes), purified by partition chromatography on a column of Celite No. 535 with mixtures of $CHCl_3$ and *n*-butanol (25), converted to the barium salt (26), and pyrolyzed to acetone and barium carbonate (C-3) (27). The acetone was subjected to the iodoform reaction and the resulting iodoform (C-2) oxidized to CO_2 (28). Table I presents typical data (from Experiment 5) showing recoveries of products in the fermentation and in the degradation of the fermentation acids.

[5] It is important to harvest the cells as soon as gas evolution becomes apparent. Occasionally cells with low activity on glucose have been obtained when harvesting was delayed too long.

[6] The CO_2 measured manometrically in the small flask was less than the theoretical value, although the yield from the alkali of the large flask was always close to theoretical (see Table I).

[7] The lactic acid may be purified by preparation of the guanidine salt (23) or by chromatography (24), but this has not been found necessary.

Degradation of Glucose by L. casei—The fermentation of glucose by *L. casei* and the degradation of the resulting lactic acid have been described by Wood *et al.* (18). By this method glucose carbons 1 and 6, 2 and 5, and 3 and 4 are obtained in pairs. Generally an 85 per cent yield of lactate was obtained in the ether extract, and the degradation of this acid resulted in yields similar to that shown for the *L. mesenteroides* fermentation in Table I.

TABLE I

L. mesenteroides Fermentation and Degradation Data (Experiment 5)

For details of the procedures see the text.

	mmole
Glucose in flask	1.00
CO_2 recovered (C-1)	0.900
Acetate from EtOH oxidation	0.925
Lactate in ether extract	0.811
Barium acetate pyrolyzed	0.337
CO_2 from CHI_3 (C-2)	0.264
" " $BaCO_3$ (C-3)	0.330
Lactate decarboxylated	0.600
CO_2 from COOH(C-4)	0.580
" " HCOOH (C-5)	0.302
" " CHI_3 (C-6)	0.295

TABLE II

Analytical Data on Gluconate Oxidation (Experiment 1)

For details of the procedures see the text.

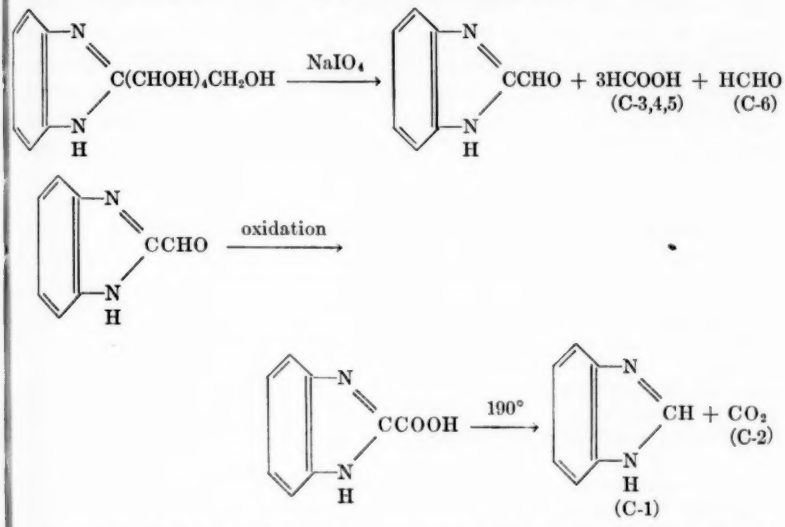
	mmoles
Potassium gluconate submitted to oxidation	1.02
$NaIO_4$ utilized (theoretical, 5 mmoles $NaIO_4$ per mmole gluconate)	4.70
CO_2 (C-1)	0.905
" from HCOOH (C-2,3,4,5)	2.97
" " HCHO (C-6)	0.630

Preparation and Degradation of Gluconate—Glucose was oxidized to potassium gluconate with potassium hypoiodite in methanol (29) and recrystallized from water by the addition of methanol. The gluconate was degraded by the following reaction as described for ribonate (26), except that 7.1 mmoles of sodium periodate were used per millimole of gluconate and 7.1 ml. of 2 M $NaAsO_2$ were used to destroy the unused periodate.

$$\underset{}{CH_2OH(CHOH)_4COOH} \xrightarrow{NaIO_4} \underset{(C\text{-}6)}{HCHO} + \underset{(C\text{-}2,3,4,5)}{4HCOOH} + \underset{(C\text{-}1)}{CO_2}$$

The analytical data for this degradation as determined in Experiment 1 are presented in Table II.

Preparation and Degradation of Glucobenzimidazole—Glucobenzimidazole was prepared from potassium gluconate by the method of Moore and Link (29) and degraded in general as described by Huebner *et al.* (30). The degradation was carried out by the accompanying reactions.



1 mmole of glucobenzimidazole was treated with 8 mmoles of $NaIO_4$ in a final volume of 50 ml. for 30 minutes at room temperature. 5 ml. of a saturated solution of $NaHCO_3$ were then added, and, after 30 minutes at room temperature, the precipitated 2-benzimidazolealdehyde was removed by centrifugation. The HCOOH (C-3,4,5) and HCHO (C-6) in the filtrate were separately oxidized to CO_2 (26). The 2-benzimidazolealdehyde was washed once with water and was oxidized to 2-benzimidazolecarboxylic acid with alkaline $KMnO_4$ (31) or with alkaline I_2 (32).

When $KMnO_4$ was used, the aldehyde was suspended in 10 ml. of 0.1 N NaOH, and, with continuous stirring, a saturated solution of $KMnO_4$ was added dropwise until the purple color persisted. After the addition of 0.75 ml. of 2 N H_2SO_4 the solution was decolorized with a saturated solution of $NaHSO_3$. The crude 2-benzimidazolecarboxylic acid was removed by centrifugation and recrystallized by extraction of the precipitate with hot saturated $NaHCO_3$, followed by acidification with 2 N H_2SO_4 added slowly with stirring. The decomposition point of 172°, uncorrected, was not depressed by mixture with an authentic sample.

When the oxidation was carried out with alkaline I_2, the aldehyde was dissolved in 20 ml. of 1 N NaOH and 23 ml. of 0.1 M I_2 (per millimole of aldehyde) were added with stirring. After 2 hours at room temperature, the solution was acidified with H_2SO_4, the excess I_2 destroyed with $Na_2S_2O_3$, and the 2-benzimidazolecarboxylic acid recrystallized as above.

The 2-benzimidazolecarboxylic acid was decarboxylated to CO_2 (C-2) and benzimidazole (C-1) (30) by heating in a glass tube in an electric furnace at 190°. CO_2-free N_2 was continually passed through the tube and through two successive traps; the first, in a CO_2-Cellosolve mixture, retained the benzimidazole, while the second, containing carbonate-free NaOH, trapped the CO_2. The benzimidazole was characterized by an uncorrected melting point of 171° (not depressed by mixture with an au-

TABLE III

Analytical Data on Glucobenzimidazole Degradation (Experiment 1)

For details of the procedures see the text.

	mmoles
Glucobenzimidazole submitted to oxidation	0.900
$NaIO_4$ utilized (theoretical, 4 mmoles $NaIO_4$ per mmole glucobenzimidazole)	3.38
CO_2 from HCOOH (C-3,4,5)	2.16
" " HCHO (C-6)	0.584
2-Benzimidazolecarboxylic acid isolated	0.495
" " decarboxylated	0.316
CO_2 from decarboxylation (C-2)	0.265
Benzimidazole isolated (C-1)	0.249

thentic sample) and an ultraviolet spectrum in alcohol corresponding to that reported by Steck *et al.* (33). Analytical data for this degradation (Experiment 1) are presented in Table III.

Determination of Radioactivity—The gas phase counting method of Bernstein and Ballentine (34) was used to determine radioactivity, and the results are expressed as counts per minute per millimole of carbon. All determinations which are considered to be significant were counted sufficiently long to give a standard error of 3 per cent or less in the radioactivity assay, except as noted in Table IV. Glucose, potassium gluconate, and benzimidazole were burned to CO_2 (35) for assay of their specific activities.

Results

The samples of glucose-C^{14} analyzed in Experiments 1 and 2, Table IV, contain their major activities in carbons 3 and 4. By the *L. mesenteroides* fermentation the activity was essentially equal in these two carbons. The results with *L. casei* confirm the levels of C^{14} in carbons 3 and 4 in Experi-

TABLE IV

Comparison of C^{14} Distribution in Glucose Determined by Chemical and Bacterial Degradation

For details of the procedures see the text. The samples were counted at the levels indicated (*i.e.*, without dilution). The results are in counts per minute per millimole of carbon.

	Glucose-3,4-C^{14}		Glucose-C^{14}	Glucose-2-C^{14}	Glucose-6-C^{14}
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Fermentations					
L. mesenteroides, C-1	69	2,630	20,300	25	10
C-2	60	5,190	7,080	13,400	320
C-3	2550	242,000	4,270	143	100
C-4	2560	253,000	4,150	0	0
C-5	60	4,880	7,650	56	160
C-6	9	750	21,800	0	15,400
Average	885	84,700	10,900	2,270	2,670
L. casei, C-1,6	49		18,500		
C-2,5	57		6,920		
C-3,4	2560		4,140		
Average	888		9,850		
Total carbon					
Glucose, C-1–6	868	86,500			
Gluconate, C-1–6	871	84,200		2,350	2,480
$NaIO_4$ oxidation					
Gluconate, C-1	71	2,600	19,700		
C-2,3,4,5	1290	124,000	5,950		
C-6	23	790	21,700		
Average	875	83,200	10,900		
Glucobenzimidazole, C-1	66*	2,570	20,700		
C-2	70†	5,080	7,280		
C-3,4,5	1670	168,000	5,550		
C-6	33	720	21,000		
Average	863	85,400	10,900		

* Standard error of this determination, ±13 per cent.

† Standard error of this determination, ±7 per cent.

ment 1, and the results of all degradations are in close agreement. In the periodate oxidation of gluconate, carbons 2, 3, 4, and 5 averaged 1290 and 124,000 c.p.m. per mmole of carbon, while by the *L. mesenteroides* fermentation these carbons averaged 1310 and 126,000, respectively. In the degradation of glucobenzimidazole, carbons 3, 4, and 5 averaged 1670 and 168,000 c.p.m. per mmole and by *Leuconostoc* fermentation, 1720 and 167,000. In Experiments 1 and 2, the activities of carbons 2 and 5 were approximately equal (*e.g.* in Experiment 2, carbon 2 contained 5190 or 5080 and carbon 5, 4880). The levels of C^{14} found in carbons 1 and 6 were unequal (carbon 1 = 2630, carbon 6 = 750 in Experiment 2).

In the sample of glucose-C^{14} used in Experiment 3, the average of carbons 2, 3, 4, and 5 was 5950 c.p.m. per mmole of carbon by periodate oxidation of gluconate, 5980 by degradation of glucobenzimidazole, and 5790 by *Leuconostoc* fermentation. Carbons 3, 4, and 5 had an average level of 5550 by glucobenzimidazole degradation and 5360 by fermentation with *L. mesenteroides*. The separate values obtained for carbons 1, 2, and 6 by the *L. mesenteroides* fermentation were confirmed by the chemical results, and by all methods the molecule was found to be symmetrically labeled. The data for carbons 3 and 4 with *L. casei* coincide with the results by the other methods, but the values for carbons 1,6 and 2,5 were found to be somewhat lower than indicated by the other procedures. The *L. casei* fermentation was repeated twice with similar results.

The data obtained in Experiments 4 and 5 with glucose-2-C^{14} and glucose-6-C^{14} demonstrate that there is no serious randomization of C^{14} from carbon 2 and carbon 6 in the *L. mesenteroides* degradation.

DISCUSSION

The pattern of C^{14} in glucose has generally been determined by one of the following methods of degradation or by modification thereof: the *L. casei* degradation (18), the yeast fermentation (36), or the chemical methods of Topper and Hastings (37) and of Abraham *et al.* (38). In these fermentative degradations carbons 1,6, 2,5, and 3,4 are obtained in pairs. The chemical degradations on the other hand yield some of the individual carbons; *e.g.*, carbons 1, 2, and 6 can be obtained by the method of Topper and Hastings, while that of Abraham *et al.* gives carbons 1, 3, 4, and 6. In the latter degradation the values for all 6 carbons can be obtained by difference without any assumptions relative to the labeling in the glucose. The advantage of the *L. mesenteroides* degradation is that all 6 carbons of glucose can conveniently be obtained as separate fractions, thus avoiding the errors that may occur when values are obtained by difference. This can be done on as little as 0.5 mmole. The results from this investigation together with those of Gunsalus and Gibbs (2) and Horecker *et al.* (15) prove

that cross-contamination in this degradation is low, probably no more than 2 per cent.

There has existed some question as to whether glycogen from the livers of rats given $NaHC^{14}O_3$ actually contains any activity in positions other than carbons 3 and 4. This has resulted because of uncertainty concerning the *L. casei* degradation. Gibbs *et al.* (39) found in *L. casei* degradations of glucose-1-C^{14} that 3 per cent of the isotope appeared in the carboxyl carbon of the lactic acid, while from glucose-3,4-C^{14} (obtained from the glycogen of rats which had received $NaHC^{14}O_3$) 2 per cent of the label was in the α and 3 per cent in the β position of the lactic acid. HBr oxidation of the glucose-3,4-C^{14} sample indicated, however, only 0.3 per cent of the total activity in carbon 1. From these data, Gibbs *et al.* suggested that the activities reported in positions other than carbons 3 and 4 of glucose-3,4-C^{14} obtained from "$NaHC^{14}O_3$-labeled" glycogen were a result of randomization of C^{14} in the *L. casei* fermentation and were not true activities in the glycogen *per se*. The combination of chemical and bacterial procedures used in Experiments 1 and 2 of the present investigation make it probable that the activities shown in carbons 1, 2, 5, and 6 are real. It seems unlikely from the data above and those of Roseman (40) that carbons 1, 2, and 6 would be significantly contaminated in the glucobenzimidazole degradation or carbon 6 in the gluconate degradation.[8] Although Topper and Hastings (37), using liver slices and $NaHC^{14}O_3$, found no activity in positions other than 3 and 4 by chemical degradation, it is quite possible that less randomization of tracer occurs during the synthesis of liver glycogen in slices than occurs in this synthesis *in vivo*.[9] The pattern of tracer under the latter conditions is very likely affected by the metabolic pathways of all the other tissues and organs.

In using the tracer pattern of liver glycogen as an indicator of metabolism, the assumption is made that the polysaccharide reflects the distribution of C^{14} in the hexose monophosphate pool. Since the oxidative cycle of Fig. 1 would introduce C^{14} into carbons 2 and 1 from 3 and 4 and since little C^{14} is found in these positions relative to that in carbons 3 and 4 when $NaHC^{14}O_3$ is administered, it appears that only a small fraction of the total number of molecules of the hexose monophosphate pool originated

[8] The data of Eisenberg (41) suggest that carbon 6 of potassium gluconate obtained by oxidation with periodate as described above is contaminated by a maximum of 5 per cent from the remaining 5 carbons. Carbon 6 obtained from potassium gluconate-6-C^{14} had a specific activity of 95 per cent, as high as was expected from the specific activity of the entire molecule.

[9] It is interesting that Bloom *et al.* (8) concluded that liver slices used the alternate pathway for most of the oxidation of glucose to CO_2; yet the labeling of the glycogen as found by Topper and Hastings (37) gives no indication of the occurrence of this pathway.

via the cycle in Fig. 1. This interpretation is not necessarily contradictory to the results of Bloom *et al.* (8) and to their conclusion that at least 75 per cent of the CO_2 arising from glucose in liver slices is formed via the hexose monophosphate oxidative cycle. These workers investigated the oxidation of glucose to CO_2, but not its total metabolism. It seems likely that a major activity of the liver is to synthesize and elaborate glucose and that the total number of glucose molecules oxidized is relatively small compared to the total number participating in other reactions. Thus the oxidative cycle (Fig. 1), though possibly playing a major rôle in the oxidation of this small amount of glucose, would have only a minor effect on the labeling of the hexose monophosphate and the glycogen. Nevertheless, even though the activity was low, the tracer pattern in carbons 1, 2, 5, and 6 should provide information on the oxidative cycle of Fig. 1, since the C^{14} in these positions presumably would reflect its operation rather than that of the Embden-Meyerhof glycolytic mechanism and the tricarboxylic acid cycle. When the data of Experiment 2 are examined from this viewpoint, the distribution pattern is not in full accord with Fig. 1. The activities in carbons 1, 2, 5, and 6 in this experiment were certainly sufficient to be measured quite accurately. Although the activities in carbons 1 and 2 could be accounted for by the operation of the cycle of Fig. 1, carbon 2 being higher than carbon 1, the activities of carbons 5 and 6 are not accounted for by this pathway, since it does not randomize tracer from carbons 3 and 4 into these positions.[10] Randomization of the tracer from carbon 2 into 5 by the triosephosphate isomerase reaction is not a likely explanation for the activity in carbon 5, since this should be accompanied by a similar randomization of C^{14} from carbon 1 into 6 (in Experiment 1, C-1 = 69, C-6 = 9; in Experiment 2, C-1 = 2630, C-6 = 750).

There appear to be at least three possible explanations for the present results: (*a*) the alternate pathway does not occur as depicted in Fig. 1, or another pathway occurs simultaneously which randomizes C^{14} into position 5; (*b*) the pools of hexose monophosphate of the glycolytic and oxidative pathways do not mix in the liver, and the tracer pattern of the glycogen isolated does not mirror that of the "oxidative" hexose monophosphate pool; or (*c*) the activity of the cycle is not manifested in the tracer pattern of the glycogen, because only a few of the molecules of hexose monophosphate converted to the polysaccharide are derived from the cycle of Fig. 1 relative to the number formed by reversal of the glycolytic reactions.

It is of interest that a similar low labeling in positions other than 3 and 4

[10] It does not seem likely that the determination of carbon 5 was in error because of cross-contamination of carbon 5 from carbons 3 and 4 during the *L. mesenteroides* degradation, since carbons 2 and 5 by the *L. casei* degradation equaled carbon 2 plus 5 by the *L. mesenteroides* degradation in Experiment 1.

of liver glycogen was obtained by Feil and Lorber (42) in experiments of longer duration on fed animals. In these experiments pellets of $CaC^{14}O_3$ were implanted intraperitoneally in rats for 7 days. Even under these conditions, in which the oxidative pathway might play a relatively more dominant rôle than it does during glycogen deposition after a fast, there was no substantial evidence for the occurrence of the pathway diagramed in Fig. 1.

Muntz and Murphy[11] in this laboratory, considered that under conditions of physiological control the oxidative pathway in liver might be much less active than in liver slices. To test this point they injected glucose-1-C^{14} and glucose-6-C^{14} separately into the portal vein of anesthetized rats and determined the activity of the tissue CO_2-HCO_3^- of the liver 1.5 minutes after the injection. The level of C^{14} of the CO_2 was much higher from glucose-1-C^{14} than from glucose-6-C^{14}. Thus their results obtained *in vivo* were similar to those of Bloom and Stetten (9) obtained *in vitro*.

With regard to the calculation (8, 9, 11)[1] of the relative rôles of the alternate pathways and of the Embden-Meyerhof-tricarboxylic acid pathways in the oxidation of glucose to CO_2, it should be noted that it is based on a comparison of the *yield of* $C^{14}O_2$ from the glucose-1-C^{14} with that from glucose-6-C^{14} (9). The larger yield of $C^{14}O_2$ from the 1 position compared to that from the 6 position is taken as an indication of the greater relative rôle of the alternate pathway. Calculations made on this basis may not give a true indication of the *relative rôles* of the pathways in the oxidation of glucose to CO_2. In the alternate pathways, perhaps, only three reactions are necessary to yield CO_2 from carbon 1 of glucose, whereas via glycolysis carbon 6 must pass through all the steps of glycolysis, go through the tricarboxylic acid cycle, and recycle before it appears as CO_2. Throughout the glycolytic reactions and the tricarboxylic acid cycle there is interaction with the metabolism of fat and protein, and the possibilities for the conversion of the C^{14} from carbon 6 of glucose to other products are thus many fold compared to that of carbon 1 by the alternate pathway.

Lewis *et al.* (10) approached the problem in a different manner by using glucose-1-C^{14} and glucose-U-C^{14} and determining the activity in acetate, lactate, and pyruvate. This approach eliminates certain of the difficulties inherent in the use of CO_2 as an indicator. They assumed that these three compounds would be unlabeled from glucose-1-C^{14} by the alternate pathway, since the C^{14} is lost in the initial decarboxylation. Therefore the extent that the activity of the acetate or the other compounds fell below that predicted for glycolysis of glucose-1-C^{14} was taken as a measure of the alternate pathway. The glucose-U-C^{14} was used to obtain a correction for

[11] Muntz, J. A., and Murphy, J. R., unpublished.

dilution that occurred by endogenous metabolism. One of the assumptions made in this calculation is that the compound selected for assay is formed from glucose only in the pathways being tested. For example, if pyruvate is formed by a combination of a 2-carbon and a 1-carbon unit, of which the latter is in equilibrium with $C^{14}O_2$ (formed via the alternate pathway), then the calculated value for the "glycolytic" contribution would be greater than its true value. Similarly an error might result if dihydroxyacetone phosphate and glyceraldehyde phosphate were not brought to "isotopic equivalence" by triosephosphate isomerase. That this may indeed be the case is indicated by Schambye *et al.* (43), who found that the glucose of liver glycogen is not symmetrically labeled when glycerol-C^{14} is fed to rats. They have concluded that the triosephosphate isomerase does not bring the two triose phosphates to complete C^{14} equivalence. If this also occurs in the breakdown of glucose, the C^{14} of the triose phosphate from glucose-1-C^{14} might in part be used preferentially for glycerol phosphate formation, and thus the pyruvate, lactate, and acetate would be lower in activity than that expected from glycolysis, leading to an erroneously high calculated value for the alternate pathway.

In view of the uncertainties of the calculation and the finding that the labeling in carbons 1, 2, 5, and 6 (of the glycogen obtained after administration of $NaHC^{14}O_3$) was not in full agreement with predictions based on current concepts of the hexose monophosphate oxidative cycle (Fig. 1), the significance of the alternate pathway in the oxidative metabolism of glucose in the liver seems uncertain. In terms of the over-all metabolism of glucose (total number of molecules handled) the hexose monophosphate oxidative cycle probably is of little quantitative significance. This conclusion is based on the assumption that all pathways of glucose metabolism pass through hexose monophosphate, and, therefore, that the relative rôles of the pathways would be reflected in the labeling of the hexose monophosphate and therefore in the glycogen.

SUMMARY

1. The patterns of C^{14} have been determined in glucose-3,4-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, and glucose-C^{14} (with different levels of isotope in the carbons). Chemical degradations and degradations by fermentation with *Lactobacillus casei* and *Leuconostoc mesenteroides* have been compared. The results demonstrate the validity of the *L. mesenteroides* fermentation as a method for determining the tracer pattern in glucose. By this method each carbon is obtained as a separate fraction and can be determined directly on as little as 0.5 mmole of glucose.

2. Data have been presented which show that the glycogen from the livers of rats given $NaHC^{14}O_3$ has minor activity in positions other than

carbons 3 and 4. The distribution of C^{14} in carbons 1, 2, 5, and 6, however, was not in agreement with predictions based on current concepts of the hexose monophosphate oxidative cycle. In terms of the total number of glucose molecules handled by the liver the alternate pathway apparently plays but a small quantitative rôle.

Addendum—In a recent paper, Bloom and Stetten (44) reported that of the *glucose molecules* utilized by liver slices about half are catabolized via the glycolytic pathway. They indicated that the remaining glucose could have been degraded by the reactions of the hexose monophosphate oxidative route.

BIBLIOGRAPHY

1. Wood, H. G., in Wolstenholme, G. E. W., Ciba Foundation conference on isotopes in biochemistry, London, 227 (1951).
2. Gunsalus, I. C., and Gibbs, M., *J. Biol. Chem.*, **194,** 871 (1952).
3. Entner, N., and Doudoroff, M., *J. Biol. Chem.*, **196,** 853 (1952).
4. Wood, W. A., and Schwerdt, R. F., *J. Cell. and Comp. Physiol.*, **41,** suppl. 1, 165 (1953); *J. Biol. Chem.*, **206,** 625 (1954).
5. Horecker, B. L., *Brewers Digest*, **28,** 214 (1953).
6. Racker, E., in Nord, F. F., *Advances in Enzymol.*, **15,** 141 (1954).
7. Leaver, F., and Wood, H. G., *J. Cell. and Comp. Physiol.*, **41,** suppl. 1, 225 (1953).
8. Bloom, B., Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **204,** 681 (1953).
9. Bloom, B., and Stetten, D., Jr., *J. Am. Chem. Soc.*, **75,** 5446 (1953).
10. Lewis, K. F., Blumenthal, H. J., Wenner, C. E., and Weinhouse, S., *Federation Proc.*, **13,** 252 (1954).
11. Katz, J., Abraham, S., Hill, R., and Chaikoff, I. L., *J. Am. Chem. Soc.*, **76,** 2277 (1954).
12. Dickens, F., *Brit. Med. Bull.*, **9,** 105 (1953).
13. Cohen, S. S., in Greenberg, D. M., Chemical pathways of metabolism, New York, **1,** 173 (1954).
14. Racker, E., de la Haba, G., and Leder, I. G., *Arch. Biochem. and Biophys.*, **48,** 238 (1954).
15. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207,** 393 (1954).
16. Bernstein, I. A., *J. Biol. Chem.*, **205,** 317 (1953).
17. LePage, G. A., in Potter, V. R., Methods in medical research, Chicago, **1,** 337 (1948).
18. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159,** 475 (1945).
19. Williams, R. J., and Kirby, H., *Science*, **107,** 481 (1948).
20. Jeanes, A., Wise, C. S., and Dimler, R. J., *Anal. Chem.*, **23,** 415 (1951).
21. Chargaff, E., Levine, C., and Green, C., *J. Biol. Chem.*, **175,** 67 (1948).
22. Pascu, E., Mora, T. P., and Kent, P. W., *Science*, **110,** 446 (1949).
23. Packham, M. A., and Butler, G. C., *J. Biol. Chem.*, **194,** 349 (1952).
24. Swim, H. E., and Krampitz, O. L., *J. Bact.*, **67,** 419 (1954).
25. Marvel, C. S., and Rands, R. D., Jr., *J. Am. Chem. Soc.*, **72,** 2642 (1950).
26. Bernstein, I. A., *J. Biol. Chem.*, **205,** 309 (1953).
27. Grosse, A. V., and Weinhouse, S., *Science*, **104,** 402 (1946).
28. Shreeve, W. W., Leaver, F., and Siegel, I., *J. Am. Chem. Soc.*, **74,** 2404 (1952).
29. Moore, S., and Link, K. P., *J. Biol. Chem.*, **133,** 293 (1940).

30. Huebner, C. F., Lohmar, R., Dimler, R. J., Moore, S., and Link, K. P., *J. Biol. Chem.*, **159,** 503 (1945).
31. Bistrzycki, A., and Przeworski, G., *Ber. chem. Ges.*, **45,** 3483 (1912).
32. Goodwin, L. F., *J. Am. Chem. Soc.*, **42,** 39 (1920).
33. Steck, E. A., Nachod, F. C., Ewing, G. W., and Gorman, N. H., *J. Am. Chem. Soc.*, **70,** 3406 (1948).
34. Bernstein, W., and Ballentine, R., *Rev. Scient. Instruments*, **21,** 158 (1950).
35. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).
36. Barnet, H. N., and Wick, A. N., *J. Biol. Chem.*, **185,** 657 (1950).
37. Topper, Y. J., and Hastings, A. B., *J. Biol. Chem.*, **179,** 1255 (1949).
38. Abraham, S., Chaikoff, I. L., and Hassid, W. Z., *J. Biol. Chem.*, **195,** 567 (1952).
39. Gibbs, M., Dumrose, R., Bennett, F. A., and Bubeck, M. R., *J. Biol. Chem.*, **184,** 545 (1950).
40. Roseman, S., *J. Am. Chem. Soc.*, **75,** 3854 (1953).
41. Eisenberg, F., Jr., *J. Am. Chem. Soc.*, **76,** 5152 (1954).
42. Feil, G., and Lorber, V., *Proc. Soc. Exp. Biol. and Med.*, **71,** 452 (1949).
43. Schambye, P., Wood, H. G., and Popják, G., *J. Biol. Chem.*, **206,** 875 (1954).
44. Bloom, B., and Stetten, D., Jr., *J. Biol. Chem.*, **212,** 555 (1955).

STUDIES ON CARBOHYDRATE METABOLISM IN RAT LIVER SLICES

V. GLYCEROL METABOLISM IN RELATION TO OTHER SUBSTRATES IN NORMAL AND DIABETIC TISSUE*

By JAMES ASHMORE,† ALBERT E. RENOLD,‡ FRANCES B. NESBETT, and A. BAIRD HASTINGS

(From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, November 24, 1954)

In order to characterize the biochemical changes that occur in carbohydrate metabolism in diabetes, we have previously compared the metabolism of glucose-U-C^{14}, fructose-U-C^{14}, and pyruvate-2-C^{14} in liver slices from normal and diabetic rats (1, 2). In the present study the metabolism of a fourth substrate (glycerol-α-C^{14}) has been compared in liver from normal and diabetic rats. Although glycerol *per se* is not in the direct line of the glycolytic pathway, it is believed to enter this system as triose phosphate. In the light of recent experiments on fructose (3, 4), both fructose and glycerol probably enter the glycolytic system at the same point. For this reason, these two substrates have been compared both individually and in combination. The results of these studies will be compared with those previously obtained with glucose and pyruvate in order to describe further the rôle of insulin in carbohydrate metabolism.

The rôle of glycerol as a precursor of carbohydrate has been the subject of many recent investigations. Teng *et al.* (5, 6) have studied the conversion *in vitro* of glycerol-α-C^{14} and pyruvate-2-C^{14} to lactate and carbohydrate in normal rat liver and kidney tissue. The incorporation of C^{14}-labeled glycerol into carbohydrate *in vivo* has been investigated by Gidez and Karnovsky (7) in intact normal rats. Bernhard and Wagner (8) have compared the utilization of deuterium-labeled glycerol in intact normal and diabetic rats. Utilization was identical in both normal and diabetic livers, and in each case deuterium was found in the liver glycogen.

* This work was supported in part by the United States Atomic Energy Commission and the Eugene Higgins Trust through Harvard University. A preliminary report of the data was given before a meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1954.

† Schering Fellow of the Endocrine Society, 1953–54.

‡ Postdoctoral Fellow of the United States Public Health Service, 1951–53.

Materials and Methods

Animals—Male albino rats of the Wistar strain, raised in our colony, weighing between 250 and 350 gm., were used. They were fed *ad libitum* on Purina laboratory chow. Alloxan diabetes was produced by intravenous injection of alloxan monohydrate (40 mg. per kilo) in the manner previously described by Renold *et al.* (1). To determine the effect of insulin, alloxan-diabetic rats were injected with 5 units of protamine zinc insulin at 12 hour intervals, beginning 48 hours prior to sacrifice of the animal.

Medium and Substrates—A medium of the ionic composition K = 110, Mg = 20, Ca = 10, HCO_3 = 40, and Cl = 130 mmoles per liter was employed. Glycerol was used as a substrate at a concentration of 40 mmoles per liter, and fructose at 20 mmoles per liter, unless otherwise noted. The solutions were equilibrated with 95 per cent oxygen-5 per cent CO_2, giving a pH in the presence of tissue of 7.4 to 7.5. Fructose[1] was uniformly labeled in all carbon atoms with C^{14}; glycerol[1] was labeled in the α-carbon position. In some experiments in which both glycerol and fructose were present as substrate, two flasks with identical contents were used, save that in one the glycerol was labeled and in the other the fructose was labeled.

Methods—The enzyme glucose oxidase, prepared from *Penicillium notatum*, is very specific for glucose and does not act on any of the other closely related hexoses (9). Chiu and Needham (10) have used this enzyme as a specific means of determining glucose production by liver slices, calculating the glucose present from the oxygen uptake measured in a Warburg respirometer. Such a method requires 5 to 10 μmoles of glucose for sufficient accuracy. By measuring the reducing substances present in the incubation medium with the Nelson (11) colorimetric method before and after destruction of the glucose present with glucose oxidase, we have been able to estimate true glucose on 1 μmole of glucose.

0.2 ml. of the liver slice medium was incubated for 1 hour at room temperature with 0.2 ml. of glucose oxidase solution (100 mg. of Sigma glucose oxidase dissolved in 5 ml. of 0.154 M acetate buffer, pH 5.7) in a graduated centrifuge tube. The tube was rotated so that its contents formed a thin film on the wall of the vessel and the tube placed in a horizontal position during the incubation period. The mixture was deproteinized with 0.5 ml. each of the Somogyi (12) reagents, and the total volume was adjusted to 2.5 ml. 1 ml. of this solution was assayed for reducing sugar. An enzyme blank and a control consisting of the enzyme plus a known quantity of glucose were used in addition to the reagent blank. True glu-

[1] Uniformly labeled fructose was obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois; glycerol-α-C^{14} was supplied by Dr. M. L. Karnovsky of this laboratory.

cose was determined by the difference between the reducing value of the medium before and after destruction of the glucose.

When this method was employed with media in which glycerol was the only added substrate, no reducing substances other than glucose could be detected. Similarly, when differential color tests for triose (13) were applied, no reducing substances could be detected. Therefore in experiments with both fructose and glycerol as added substrates, fructose in the medium was determined by the Nelson method after destruction of the glucose with glucose oxidase. This method also permits the determination of the specific activity of the added labeled fructose at the end of the incubation. 5 ml. of the liver slice medium and 1 ml. of the glucose oxidase solution were equilibrated with 95 per cent oxygen-5 per cent CO_2 in a Scholander respirometer (14) at 30° until no further oxygen uptake was observed. The

TABLE I

Specific Activity of Fructose before and after Incubation with Rat Liver Slices

The results are expressed in counts per minute per millimole.

Initial	Final
1.15×10^6	1.20×10^6
	1.25×10^6
0.89×10^6	0.82×10^6
	1.04×10^6
1.41×10^6	1.40×10^6
	1.36×10^6

solution was deproteinized by heating in a water bath at 60° for 5 minutes, and the protein was removed by centrifugation. The phenylosazone for radio assay was formed from the supernatant fluid. The results in Table I show that the specific activity of the added fructose remains unchanged during the course of the experiment.

The specific activity of the glucose formed in the presence of added labeled fructose was determined by the method previously described by Renold *et al.* (2). Other methods involving chemical and isotopic analyses were identical with those reported by Hastings *et al.* (15) and Teng *et al.* (5).

Calculations—Initial and final glycogen concentrations, net change in glycogen content, and initial and final concentrations of glucose, fructose, and glycerol in the media were calculated from the data obtained by direct chemical analyses. The following were calculated from the isotopic and analytical data in accordance with the method previously outlined (5): (1) glycogen formation from glycerol, (2) glycogen formation from fructose,

(3) glucose production from added glycerol, (4) glucose production from added fructose, (5) CO_2 production from added glycerol, (6) CO_2 production from added fructose.

Results

Metabolism of Glycerol by Normal and Diabetic Rat Liver—Table II summarizes the incorporation *in vitro* of glycerol-C^{14} into glucose, glycogen, and CO_2 by normal and diabetic rat liver tissue. The striking defect in glycerol metabolism in the diabetic is the reduced amount of glycogen (0.4 as opposed to 6.4 μmoles per gm.) formed from this substrate. Otherwise the metabolism of glycerol appears to be essentially unchanged. The injection of insulin over a 48 hour period restores glycogen production from

TABLE II

Incorporation of C^{14} of Labeled Glycerol (40 mmoles per Liter) into Glycogen, Glucose, and CO_2 by Rat Liver Slices

All results are expressed in micromoles per gm. of wet liver per 90 minutes ± the standard error. The figures represent the results of six observations.

	Glycerol uptake	Glucose output	Glucose from glycerol	Glycogen from glycerol	CO_2 from glycerol
Normal	129 ±2.6	87 ±5.5	39 ±2.5	6.4 ±1.1	42 ±1.9
Diabetic	101 ±10	90 ±6.3	45 ±3.7	0.4 ±0.01	40 ±3.6
Diabetic + insulin	107 ±3.3	67 ±3.7	25 ±2.5	6.4 ±0.66	52 ±4.6

glycerol to the normal level. Insulin also appears to act in the diabetic to reduce by 45 per cent the amount of glucose formed from glycerol, as well as to reduce the total glucose produced by the liver slice.

Metabolism of Fructose by Normal and Diabetic Rat Liver—Data on fructose metabolism in the liver are summarized in Table III. In agreement with previous observations (2), this substrate was incorporated into glucose and oxidized to CO_2 as readily in the diabetic as in the normal, and its incorporation into liver glycogen was reduced in the diabetic liver. Glycogen formation from fructose was restored to normal in the diabetic by the injection of insulin.

Comparison of Fructose and Glycerol Metabolism—In experiments (Table IV) in which the two substrates were studied in combination, glycerol and fructose contributed equally to the glucose and glycogen produced. In the presence of added fructose, glycerol metabolism in both normal and diabetic liver slices was essentially the same as with glycerol alone in the medium.

The lower fructose utilization in these experiments (73 and 46 μmoles per gm. per 90 minutes) is accounted for by the reduced concentration of fructose present when both glycerol and fructose were substrates. When fructose was present alone and at the same concentration (10 mmoles per liter),

TABLE III

Incorporation of C^{14} of Labeled Fructose (20 mmoles per Liter) into Glycogen, Glucose, and CO_2 by Rat Liver Slices

All results are expressed in micromoles per gm. of wet liver per 90 minutes ± the standard error.

	Fructose uptake	Glucose output	Glucose from fructose	Glycogen from fructose	CO_2 from fructose
Normal (3)*	144 ±2.6	111 ±4.7	72 ±2.3	20 ±1.4	105 ±8.0
Diabetic (4)	111 ±1	167 ±8	76 ±8.3	4.6 ±1	93 ±8.4
Diabetic + insulin (3)	127 ±9.1	106 ±3.4	82 ±9.3	16.6 ±2.8	99 ±1.2

* The figures in parentheses represent the number of observations.

TABLE IV

Metabolism of Fructose (10 mmoles per Liter) and Glycerol (20 mmoles per Liter) Present Together in Medium by Rat Liver Slices

All results are expressed in micromoles per gm. of wet liver per 90 minutes ± the standard error.

	Fructose uptake	Glycerol uptake	Glucose output	Glucose from fructose	Glucose from glycerol	Glycogen from fructose	Glycogen from glycerol
Normal (4)*	73 ±4.8	103 ±7.9	85 ±5.2	32 ±11	34 ±1.0	10.3 ±1.3	12.0 ±1.6
Diabetic (8)	46 ±3.8	99 ±5.6	120 ±7.7	26 ±4.5	29 ±3.0	0.63 ±0.10	0.65 ±0.11

* The figures in parentheses represent the number of observations.

it was utilized at a rate (60 μmoles per gm. per 90 minutes) comparable to that observed in those experiments in which glycerol was also present.

Rate of Metabolism of Glycerol by Normal and Diabetic Liver—A question often raised in metabolic experiments is whether or not the products of metabolism observed at the end of the experimental period bear the same relationship to each other that they do during the entire course of the experiment. In our specific case, did the small quantity of labeled glycogen found in the diabetic liver at the end of the experimental period represent

the maximal glycogen from substrate present at any time during the 90 minute incubation? In order to investigate this point, liver slices from normal and diabetic rats were incubated with labeled glycerol for 30, 60, and 90 minutes. At the end of the incubation periods substrate utilization and conversion to glycogen and glucose were determined. The results of these studies are presented in Fig. 1. Each curve represents the mean of three experiments.

The utilization of glycerol appears to be uniform over the experimental period in both normal and diabetic liver slices. In both normal and diabetic liver slices a greater fraction of the glycerol that is utilized during

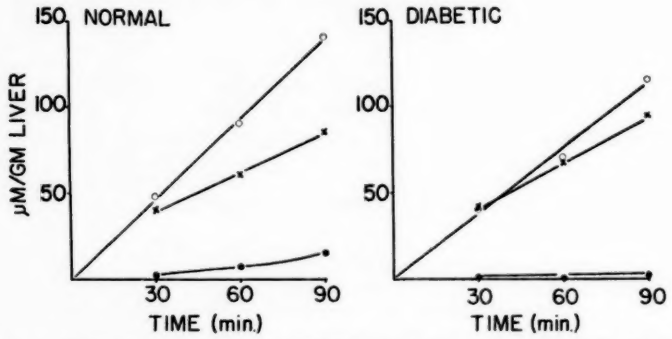


FIG. 1. Metabolism of glycerol-α-C^{14} (40 mmoles per liter) by liver slices from normal and diabetic rats. Glycerol utilization (○) and the incorporation of this substrate into glucose (×) and glycogen (●) determined at 30, 60, and 90 minutes. Each curve represents the mean of three experiments.

the first 30 minutes is converted to glucose than during the last 60 minutes of incubation. Nevertheless, the increased conversion of glycerol to glucose and decreased formation of glycogen from glycerol in the diabetic compared with normal are apparent whether the incubation period is 60 or 90 minutes. It would therefore appear that conclusions reached from 90 minute observations would be the same for the metabolism of glycerol by liver slices over the 30 to 90 minute experimental period.

DISCUSSION

It has been proposed (3, 4) that fructose, after initial phosphorylation to fructose-1-phosphate, is split to glyceraldehyde and phosphodihydroxyacetone. If this is true, fructose and glycerol would thus both enter the glycolytic system at the triose stage, though arriving by different routes. The marked similarity in the metabolism of these two substrates when

present separately or in combination does indeed suggest that their pathways in the glycolytic system are identical. Furthermore, the metabolism of each substrate was found to be altered equally in the diabetic state.

From a comparison of the metabolism of these substrates, glycerol and fructose, with that of glucose and pyruvate in normal and diabetic liver (1), it would appear that glucose is the only one of these substrates showing

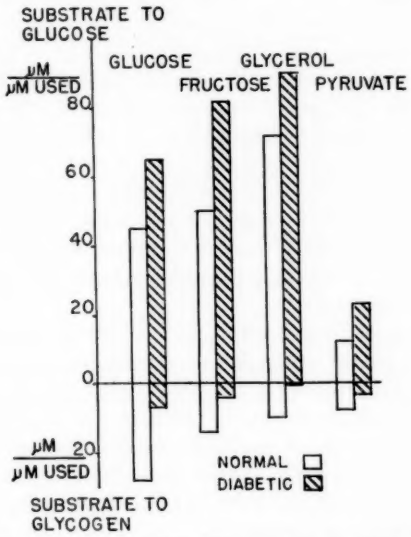


FIG. 2. Metabolism of glucose-U-C^{14} (20 mmoles per liter), fructose-U-C^{14} (20 mmoles per liter), glycerol-α-C^{14} (40 mmoles per liter), and pyruvate-2-C^{14} (40 mmoles per liter) by liver slices from normal and diabetic rats. The height of the bar above the line represents the per cent (micromoles of substrate per micromole used) of the substrate used that was incorporated into glucose during the 90 minute incubation period. The height of the bar below the line represents the micromoles of substrate per micromole used that was incorporated into liver glycogen.

a marked reduction in utilization in the diabetic. Renold *et al.* (2) have estimated that glucose phosphorylation was decreased to approximately 20 per cent of the normal value in the diabetic liver slice. It is presumably at this stage, *i.e.* either phosphorylation or entry into the cell, that metabolism of glucose is materially altered in diabetes. Injection of insulin (16) over a 48 hour period restores the ability of the diabetic liver to utilize glucose.

There appears to be no impairment in the ability of these substrates to form glucose and CO_2 in the diabetic liver, but in every case the conver-

sion of substrate to glycogen is greatly reduced in diabetes. In order to consider the fate of each substrate independently of the amount utilized, the per cent of the substrate utilized that appears as glucose or glycogen has been plotted in Fig. 2. All four substrates exhibit the same change in metabolic pattern in the diabetic state; *i.e.*, the per cent of the substrate incorporated into glycogen is reduced in the diabetic and is accompanied by an increase in the per cent of substrate converted to glucose.

The per cent of substrate utilized for synthesis of carbohydrate (glucose + glycogen) is increased over normal in the diabetic. This means that in the diabetic there must exist a diversion of substrate in the glycolytic pathway toward an increased formation of glucose at the expense of formation of glycogen. Both glucose and glycogen have glucose-6-phosphate as a common intermediate. In the diabetic an increase in liver glucose-6-phosphatase in relation to other enzymes between glucose-6-phosphate and glycogen would be consistent with the observation of a diversion of substrate carbons from glycogen formation and toward glucose formation. An increase in liver glucose-6-phosphatase has, indeed, been observed to occur in alloxan-diabetic rats (17).

In the diabetic liver, carbohydrate metabolism is altered to the extent that there is a decreased utilization of glucose, a decreased formation of glycogen from glucose, fructose, glycerol, and pyruvate, and an increased diversion of carbohydrate to glucose. These conditions can be returned to normal by the injection of insulin *in vivo* for 48 hours prior to the sacrifice of the animal. In these studies, no striking alteration in carbohydrate metabolism other than at the glucose-6-phosphate level has been observed in the absence of insulin.

SUMMARY

The metabolism of glycerol-α-C^{14} in liver slices from fed normal and diabetic rats has been compared with that of glucose, fructose, and pyruvate. All four substrates exhibit the same general metabolic pattern. In addition to a diminished utilization of glucose, liver slices from diabetic rats produced more glucose and less glycogen from each of these substrates than did normal liver tissue. The metabolism of diabetic liver tissue could be returned to normal by the injection of insulin prior to sacrifice of the animal.

BIBLIOGRAPHY

1. Renold, A. E., Teng, C.-T., Nesbett, F. B., and Hastings, A. B., *J. Biol. Chem.*, **204,** 533 (1953).
2. Renold, A. E., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **209,** 687 (1954).
3. Hers, H. G., and Kusaka, T., *Biochim. et biophys. acta*, **11,** 427 (1953).
4. Leuthardt, F., Testa, E., and Wolf, H. P., *Helv. chim. acta*, **36,** 227 (1953).

5. Teng, C.-T., Karnovsky, M. L., Landau, B. R., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **202,** 705 (1953).
6. Teng, C. T., *Arch. Biochem. and Biophys.*, **48,** 409 (1954).
7. Gidez, L. I., and Karnovsky, M. L., *J. Biol. Chem.*, **206,** 229 (1954).
8. Bernhard, K., and Wagner, H., *Helv. chim. acta*, **35,** 330 (1952).
9. Keilin, D., and Hartree, E. F., *Biochem. J.*, **42,** 221 (1948).
10. Chiu, C. Y., and Needham, D. M., *Biochem. J.*, **46,** 114 (1950).
11. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
12. Somogyi, M., *J. Biol. Chem.*, **160,** 69 (1945).
13. Hers, H. G., Beaufays, H., and de Duve, C., *Biochim. et biophys. acta*, **11,** 416 (1953).
14. Scholander, P. F., Claff, L. C., Andrews, J. R., and Wallach, D. F., *J. Gen. Physiol.*, **35,** 375 (1952).
15. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, **194,** 69 (1952).
16. Renold, A. E., Hastings, A. B., Nesbett, F. B., and Ashmore, J., *J. Biol. Chem.*, **213,** 135 (1955).
17. Ashmore, J., Hastings, A. B., and Nesbett, F. B., *Proc. Nat. Acad. Sc.*, **40,** 673 (1954).

A PROTEASE PRESENT IN EXTRACTS OF A LYMPHATIC ORGAN*

By E. L. HESS, MILDRED CAMPBELL, and AILENE HERRANEN

(From the Rheumatic Fever Research Institute, Northwestern University Medical School, Chicago, Illinois)

(Received for publication, September 16, 1954)

The presence in animal tissues of enzymes which hydrolyze proteins under acid conditions has long been recognized. Aside from the demonstration of their existence, very little is known about these enzymes. A few pages in the recent review by Smith (1) adequately cover the work of the first half of the century. Only in the most recent work (2–4) have some of the unusual properties of these enzymes been noted.

This report will describe some of the properties of a protease present in extracts of bovine palatine tonsils, which hydrolyzes proteins under acid conditions. In addition, the usefulness of the electrophoretic method for evaluating the products of the hydrolysis process will be demonstrated.

EXPERIMENTAL

Materials—Bovine tonsil extracts were prepared as described in an earlier publication (5). The enzyme was found to be concentrated in Fraction 4.2S (Supernatant C in the publication referred to). This crude fraction (No. 4.2S) unless otherwise specified was used as the enzyme in the work to be described. The electrophoretic pattern given by Fraction 4.2S has been published (6).[1] Armour's crystalline bovine serum albumin (BSA) was used as a substrate.

Methods—Proteolytic activity was followed both spectrophotometrically and electrophoretically. In the spectrophotometric method, 1 ml. of enzyme solution was added to 4 ml. of a 1 per cent solution of BSA in buffer. The solutions were incubated for 120 minutes at 37° without shaking. After incubation, 5 ml. of 20 per cent trichloroacetic acid (TCA) were added. Blanks containing substrate without enzyme and enzyme without substrate were included. Supernatant solutions, after centrifugation, were diluted 1:5 with water and read at 280 mμ in a model DU Beckman spectrophotometer against 2 per cent TCA. Sodium phosphate buffers at pH 3.0 and an ionic strength of 0.10 were used routinely for spectrophotometric

* Supported in part by a grant from the United States Atomic Energy Commission, contract No. AT(11-1)-89 with Northwestern University.

[1] Fraction 4.2S contains 30 per cent albumin and 70 per cent other proteins having mobilities distributed from 2.0 to 6.0 cm.^{2} volt^{-1} sec.$^{-1}$.

XUM

studies. When metal ions were added to the systems, monochloroacetic acid buffer was employed. Glycine-hydrochloric acid was used for buffers below pH 3 and acetate for buffers in the pH range 4 to 6.

In the electrophoretic studies, 150 mg. of albumin and 15 mg. of the tonsil fraction were dissolved in 15 ml. of phosphate buffer, μ 0.10, at pH 3.0, and allowed to stand at 4°. After an appropriate interval of time the solution was adjusted to pH 8.5 and dialyzed for 48 hours against several changes of Veronal buffer, μ 0.10, pH 8.6. The solution was then run in a conventional Tiselius assembly equipped with a Philpot-Svensson schlieren optical arrangement as described previously (5).

The refractive index increment $(n_s - n_0)/c$ of BSA in Veronal, $\mu = 0.10$, at 1° is 0.002014 for $\lambda = 5780$ A (7). The concentration of the albumin remaining in solution at the time of the experiment was calculated from the electrophoretic pattern (8). It was assumed that the refractive index increment of the various fragments was the same as that of the parent albumin molecule. This assumption is consistent with what is known about the refractive index increment of peptides and purified simple proteins (7, 9).

Results

Hydrolysis of BSA—Electrophoretic studies not only showed the disappearance of BSA, but revealed that the albumin molecule was split into fragments possessing characteristic electrokinetic properties. In Fig. 1 the patterns of BSA prior to hydrolysis and at various stages of hydrolysis can be seen. Fig. 1, *A* demonstrates the stability of BSA at pH 3 in phosphate buffer; Fig. 1, *B* shows that in the presence of Fraction 4.2S no degradation of BSA occurs at pH 8.6 in Veronal buffer, μ 0.10.

At least four fragments result from the hydrolysis of BSA, as can be seen in *C* and *D* (Fig. 1). These fragments accumulate as the BSA disappears from the solution. Three of the fragments have distinct and reproducible electrophoretic mobilities, whereas the fourth fragment does not appear to move in the electric field. We have been able to isolate the fourth component (*E*), which does not migrate at pH 4.7 in an electric field and, therefore, apparently carries virtually no electric charge. It is of interest that the ultraviolet absorption pattern of this substance showed no peak at 280 mμ. This observation, combined with an extinction coefficient $E^{1\%}_{280} \sim 2.0$ suggests that very little tyrosine and tryptophan can be present in the molecule. The fragment is soluble in saturated ammonium sulfate and does not dialyze through Visking casing. It would appear that this fragment consists chiefly of monoaminomonocarboxyamino acids.

There were fragments formed during hydrolysis which were small enough to pass through Visking casing. It was calculated from the electrophoretic

pattern that, after 24 hours at 2° and at pH 3.0 in sodium phosphate buffer, μ 0.10, 78 per cent of the original material in solution remained non-dialyz-

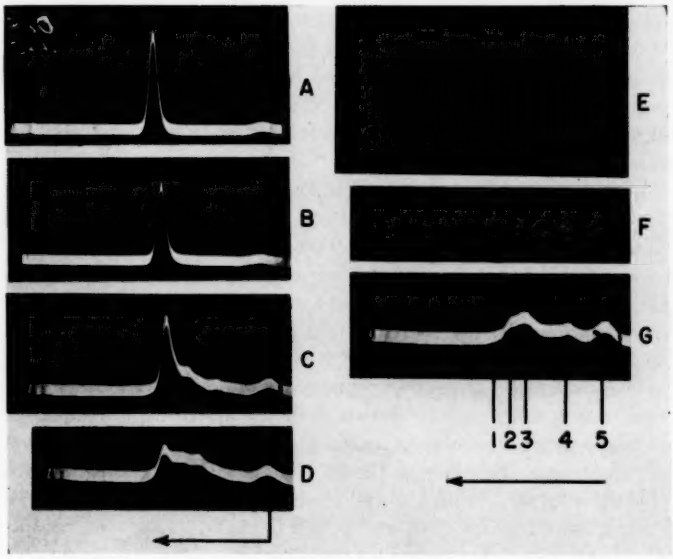


FIG. 1. Electrophoretic patterns showing the hydrolysis of bovine albumin. *A*, 1 per cent solution of BSA without enzyme, but allowed to stand at pH 3 in phosphate of μ 0.10 for 48 hours prior to dialysis against Veronal; *B*, 1 per cent solution of BSA + 0.1 per cent crude enzyme (Fraction 4.2S) dissolved directly into Veronal buffer and maintained at pH 8.6; *C*, 1 per cent solution of BSA + 0.1 per cent crude enzyme (Fraction 4.2S) allowed to stand 24 hours at pH 3.0 in phosphate of μ 0.10 at 5° prior to dialysis against Veronal buffer; *D*, same as *C* except for having stood 72 hours at pH 3.0 in phosphate of μ 0.10 at 5° prior to dialysis against Veronal; *E*, slightly charged fragment; *F*, unhydrolyzed crude fraction of tonsil albumin; *G*, same mixture after 48 hours at 5° in phosphate of μ 0.10, pH 3.0. All patterns are of descending boundaries after 120 minutes under a potential gradient of 6.4 volt cm.$^{-1}$ in Veronal buffer of μ 0.10, pH 8.6. Diagonal slit angle 20° in *A*, 15° in *B*, 30° in *C*, *D*, *E*, and *F*, 40° in *G*. Original protein concentration (weight per volume) 1 per cent in *A*, 1.1 per cent in *B*, *C*, *D*, *F*, and *G*, 2 per cent in *E*. In the case of *E* the position of the initial boundary with relation to the boundary position after 120 minutes is shown for comparison. *1* to *5*, individual components.

able. Of this, it was calculated that not more than 57 per cent was BSA. Therefore 56 per cent of the original BSA was hydrolyzed. After standing 72 hours under the conditions stated above, about 30 per cent of the contents of the solution passed through Visking casing. Of the remaining 70 per cent only 27 per cent was unhydrolyzed BSA. Therefore 80 per cent of the albumin originally present was hydrolyzed.

Solubility Behavior of Enzyme—Fraction 4.2S[1] contains albumin which is indistinguishable from BSA (6). In the course of the separation and characterization of the tonsil albumin (6), Fraction 4.2S in the form of a dry lyophilized powder was extracted with 0.65 saturated ammonium sulfate[2] at pH 6. Enzyme activity was found in the soluble extract.

The soluble fraction (No. 65S) was brought to saturation with solid ammonium sulfate. The precipitate, removed by centrifugation, was dissolved in water, adjusted to pH 8.5 to inhibit hydrolysis, dialyzed against water, and lyophilized.

A 1 per cent solution of this precipitate gave the electrophoretic pattern seen in Fig. 1, *F*. A 1 per cent solution of the same fraction dissolved in phosphate buffer of μ 0.10 at pH 3.0 was allowed to stand at 2°. After 48 hours the pH of this solution was adjusted to 8.5 with 0.1 N sodium hydroxide, and the solution was dialyzed for 48 hours against several changes of Veronal buffer and examined in the Tiselius apparatus. The pattern obtained may be seen in Fig. 1, *G*. It was found that 75 per cent of the original concentration of protein remained non-dialyzable.

Several conclusions may be drawn from the above experiments. First, the enzyme was fairly soluble in 0.65 saturated ammonium sulfate at pH 6. Second, the tonsil albumin was almost completely hydrolyzed in 48 hours at 2° by the enzyme. Third, three products of hydrolysis were apparent which have been labeled *2*, *3*, and *5* in Fig. 1, *G*. Last, the component labeled *4* is evidently unaffected by the enzyme.

In the above fractionation, the supernatant solution remaining after saturation with ammonium sulfate was dialyzed free of salt and lyophilized. This material is the uncharged fragment (Fig. 1, *E*). The same material can also be prepared by saturating a digested serum albumin solution with ammonium sulfate. The non-dialyzable material present in the supernatant solution has the properties of the material shown in Fig. 1, *E*.

The enzyme was less soluble in 0.60 saturated ammonium sulfate at pH 4 than at pH 6. Unfortunately for purposes of separation, serum albumin behaves similarly. The enzyme was found to be insoluble in 0.70 saturated ammonium sulfate at pH 6.

Effect of pH and Ionic Strength—The effect of pH upon the rate of hydrolysis of BSA is presented in Fig. 2. As shown in Table I, the rate of hydrolysis of BSA by the enzyme appears to be a function of ionic strength. Lumry, Smith, and Glantz (10) have reported that the activity of carboxypeptidase is a function of ionic strength.

Effect of Other Substances—Reducing substances, such as cysteine and glutathione, appear to accelerate the hydrolysis of BSA. All of the metal-

[2] In this instance 70 gm. of solid ammonium sulfate dissolved in 100 ml. of water at 4°.

lic ions tested appeared to be without effect on the reaction rate at concentrations of 0.05 mole per liter. These included ferrous, manganous, mercurous, magnesium, and zinc ions. Dihydrogen phosphate, mono-

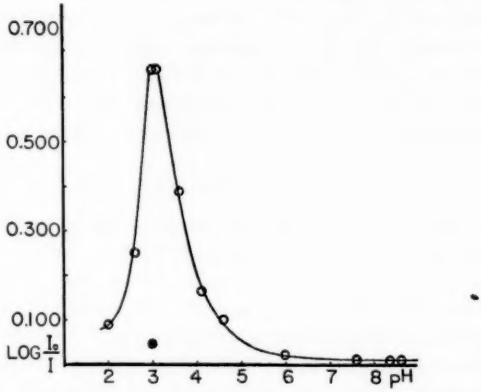


FIG. 2. Hydrolysis of BSA by tonsil enzyme as a function of pH. ○, BSA + Fraction 4.2S incubated 120 minutes at 37°; ⊗, same as above except that Fraction 4.2S solution was heated for 10 minutes at 90° prior to adding the substrate. Coagulation occurred when Fraction 4.2S was heated. Log I_0/I values represent the optical density of the supernatant solution after precipitation with 10 per cent TCA.

TABLE I

Effect of Ionic Strength upon Hydrolysis of BSA Incubated at 37° for 120 Minutes in Phosphate Buffer

Ionic strength	Log $\frac{I_0}{I}$*
0.100	0.328
0.080	0.320
0.040	0.229
0.008	0.129
0.001	0.128
0.080†	0.130

In each instance 4 ml. of a 1 per cent solution of BSA in phosphate buffer were added to 1 ml. of a 1 per cent solution of Fraction 4.2S in water. The ionic strength of the final solution is shown in the first column. The pH of each solution was 3.00 ± 0.05. All solutions were precipitated with 5 ml. of 20 per cent trichloroacetic acid after 120 minutes. The supernatant solution after centrifugation was diluted 1:5 with water and the optical density (log I_0/I) read at 280 mμ in a model DU Beckman spectrophotometer.

* The average of duplicate experiments.

† Crude enzyme only with 4 ml. of buffer in place of the BSA solution.

valent citrate, and monochloroacetate were without effect. Versene (0.1 M), diisopropylfluorophosphate (5×10^{-5} M), and Marsilid (0.01 M) did not affect the reaction. α,α'-dipyridyl at 0.10 M concentration appeared to inhibit the hydrolysis slightly. In view of the apparent accelerating effect of reducing substances, it was considered advisable to examine the possible effects of oxidation. Shaking the solution containing enzyme gave no apparent effect. The addition of hydrogen peroxide was of interest in that at concentrations greater than 0.002 M increased enzyme activity was found, while a concentration of 0.001 M caused inhibition.

DISCUSSION

This study provides evidence for the presence of a proteolytic enzyme in lymphatic organs whose maximal activity is at pH 3 when BSA is used as a substrate. Our work with lymphocytes serves to confirm the earlier findings of Dernby with leucocytes (11), inasmuch as leucocytes and lymphocytes are morphologically related structures.

The failure of metallic ions to alter the activity of the enzyme system is in accord with the findings of Morrison and Neurath (4) who reported similar results with erythrocyte proteinase III. It is of interest that Morrison and Neurath have observed similar behavior towards metallic ions by a proteolytic enzyme present in leucocytes whose maximal activity was found at low pH values.[3]

The solubility of the tonsil protease in ammonium sulfate solutions is similar to that of a beef spleen enzyme as reported by Tallan, Jones, and Fruton (2). These workers found beef spleen cathepsin C in the fraction soluble in 0.45 and insoluble in 0.70 saturated ammonium sulfate at pH 7.0. The activity of beef spleen cathepsin C is enhanced by the presence of cysteine (2), another property possessed in common with the tonsil protease. Tallan *et al.* also reported (2) that manganese sulfate inhibited cathepsin C activity. Manganese ions appeared to be without effect on the activity of the crude tonsil enzyme.

Fig. 1, *B* deserves special consideration, since it demonstrates a point often overlooked. This pattern is indistinguishable from that in *A*; yet 7 per cent of material other than BSA was known to be in solution. In addition to the 150 mg. of BSA, the solution contained 15 mg. of Fraction 4.2S. The contaminating substances[1] which possess mobilities differing from that of BSA are not evident in the pattern.

Unless one assumes that the four new components appearing in Fig. 1, *D* represent four steps or four types of denaturation of the parent albumin

[3] Personal communication from Professor H. Neurath.

molecule, which seems unlikely, one must conclude that several non-dialyzable intermediates are produced when the albumin molecule is hydrolyzed by the tonsil enzyme. Furthermore, this conclusion is in accord with the results of numerous other workers (12–20) who have used proteins other than BSA as substrates for a variety of proteases. Preliminary studies indicate the feasibility of separation and characterization of these fragments. Such studies would provide worth while information concerning the intramolecular structure of the albumin molecule. Of importance in this respect is the observation that one of the subunits of BSA is essentially uncharged and contains little, if any, tyrosine and tryptophan.

The isolation and characterization of both the high and the low molecular weight products are important for elucidating the mechanism of enzymatic degradation as well as for an understanding of the intramolecular structure of the substrate molecule.

That dialyzable fragments of low molecular weight which are not precipitated by 10 per cent TCA are also produced during the enzymatic digestion of proteins is well known (21, 22). It is the accumulation of these fragments which is followed in the spectrophotometric method of assay. These fragments may or may not be products of the initial reaction step.

If the extent of hydrolysis is defined, as it should be, by the fraction of BSA hydrolyzed, which can be measured by means of the electrophoretic method, the result is quite different from that obtained by measuring the accumulation of low molecular weight products. For example, it was found that, when 4 ml. of a 1 per cent solution of BSA in phosphate buffer of μ 0.10 at pH 3.0 were incubated for 120 minutes at 37° with 1 ml. of a 1 per cent solution of Fraction 4.2S, 18 per cent of the absorption at 280 mμ of the original solution remained in the supernatant solution after precipitation with 10 per cent TCA. It was observed by means of the electrophoretic method that, even when 78 per cent of the original concentration of BSA remained non-dialyzable, 56 per cent of the BSA had been hydrolyzed.

The rate of disappearance of the substrate molecule is important in kinetic studies. A direct method for obtaining such information is preferable to an indirect one. Most of the methods (1) commonly employed for following the hydrolysis of proteins depend upon the analysis of the low molecular weight products. As we have shown in this paper, the rate of disappearance of the substrate can be followed quantitatively by means of the electrophoretic method of assay. In addition, as demonstrated by Seegers *et al.* (14), Biserte and Boulanger (16), and in this paper, electrophoretic analysis offers a convenient method for detecting the high molecular weight products of the hydrolysis.

SUMMARY

1. Evidence has been presented for the occurrence in bovine palatine tonsils of a proteolytic enzyme having a maximal activity at pH 3 when BSA is used as a substrate.

2. Properties of this enzyme have been discussed.

3. Two methods for following the digestion of bovine serum albumin have been employed. By one method, based upon electrophoretic analysis, the rate of disappearance of the BSA was followed. By the other, based upon spectrophotometric procedures, the accumulation of dialyzable products of low molecular weight was determined.

4. It has been shown that both high molecular weight, non-dialyzable and low molecular weight, dialyzable products were formed.

5. The usefulness of the electrophoretic method for assaying proteolytic activity has been demonstrated. The method allows one to characterize the high molecular weight products in terms of the electrophoretic mobility. This procedure also allows one to determine quantitatively the extent of hydrolysis of the substrate.

BIBLIOGRAPHY

1. Smith, E. L., in Sumner, J. B., and Myrbäck, K., The enzymes, New York, **1**, 857 (1951).
2. Tallan, H. H., Jones, M. E., and Fruton, J. S., *J. Biol. Chem.*, **194**, 793 (1952).
3. Demole, M. J., and Milhaud, G., *Am. J. Digest. Dis. and Nutr.*, **18**, 43 (1951).
4. Morrison, W. L., and Neurath, H., *J. Biol. Chem.*, **200**, 39 (1953).
5. Hess, E. L., Ayala, W., and Herranen, A., *J. Am. Chem. Soc.*, **74**, 5410 (1952).
6. Hess, E. L., Campbell, M., and Herranen, A., *J. Am. Chem. Soc.*, **76**, 4035 (1954).
7. Perlmann, G. E., and Longsworth, L. G., *J. Am. Chem. Soc.*, **70**, 2723 (1948).
8. Svensson, H., *Ark. Kemi, Mineral. o. Geol.*, **22 A**, 57 (1946).
9. Barer, R., and Tkaczyk, S., *Nature*, **173**, 821 (1954).
10. Lumry, R., Smith, E. L., and Glantz, R. R., *J. Am. Chem. Soc.*, **73**, 4330 (1951).
11. Dernby, K. G., *J. Biol. Chem.*, **35**, 179 (1918).
12. Petermann, M. L., *J. Phys. Chem.*, **46**, 183 (1942).
13. Bridgman, W. B., *J. Am. Chem. Soc.*, **68**, 857 (1946).
14. Seegers, W. H., Nieft, M. L., and Vandenbelt, J. M., *Arch. Biochem.*, **7**, 15 (1945).
15. Boulanger, P., Biserte, G., and Swyngedauw, J., *Biochim. et biophys. acta*, **2**, 302 (1948).
16. Biserte, G., and Boulanger, P., *Bull. Soc. chim. biol.*, **35**, 1153 (1953).
17. Williams, J. W., Baldwin, R. L., Saunders, W. M., and Squire, P. G., *J. Am. Chem. Soc.*, **74**, 1542 (1952).
18. Currie, B. T., and Bull, H. B., *J. Biol. Chem.*, **193**, 29 (1951).
19. Mihályi, E., and Szent-Györgyi, A. G., *J. Biol. Chem.*, **201**, 189 (1953).
20. Kaplan, E. H., *Proc. Soc. Exp. Biol. and Med.*, **85**, 142 (1954).
21. Beloff, A., and Anfinsen, C. B., *J. Biol. Chem.*, **176**, 863 (1948).
22. Phillips, D. M. P., *Biochem. J.*, **49**, 506 (1951).

AN ESTRADIOL-INDUCED STIMULATION OF CITRATE UTILIZATION BY PLACENTA*

By CLAUDE A. VILLEE

(From the Department of Biological Chemistry, Harvard Medical School, and the Research Laboratories of the Boston Lying-In Hospital, Boston, Massachusetts)

(Received for publication, October 29, 1954)

Estradiol-17β, at a level of 4×10^{-6} M, was found to increase the oxygen consumption of slices of human endometrium incubated *in vitro* (1). Experiments with C^{14}-labeled substrates showed that estradiol stimulates the metabolism of the carbonyl carbon of pyruvate and the carboxyl carbon of acetate to carbon dioxide (2). These results suggested that estradiol is effective somewhere in the common oxidative pathway of pyruvate and acetate. Estradiol appears to have a similar effect *in vivo*: slices of endometrium in the late proliferative phase of the menstrual cycle (2nd week), when the estradiol concentration in blood and urine is at a peak, have a greater rate of oxygen consumption and metabolize substrates to carbon dioxide faster than do slices of endometrium in the early proliferative phase (1st week) (3). In further experiments, this stimulatory effect of estradiol was demonstrated in homogenates of both young and term placentae; the evidence suggested that the site of action of the estrogen lies in the tricarboxylic acid cycle between citrate and α-ketoglutarate (4).

In the present studies both homogenates and more purified preparations of placenta were used, and the effects of certain other steroids were tested. These experiments provide additional evidence that estrogens increase the conversion of citrate to α-ketoglutarate and suggest that this may be via a stimulation of a DPN[1]-linked isocitric dehydrogenase.

Materials and Methods

Term placentae obtained from normal spontaneous deliveries or from cesarean sections performed at term were taken directly to the laboratory in ice-cold saline. Homogenates and cell fractions were prepared and used immediately.

Homogenates containing 20 per cent (weight per volume) of placenta in ice-cold 0.25 M sucrose were prepared in a smooth glass homogenizer fitted with a Teflon pestle. After homogenization for 20 seconds at ice bath tem-

* This investigation was supported by grants from the Charles A. King and Marjorie King Fund, and by an Institutional Research Grant from the American Cancer Society.

[1] ATP = adenosine triphosphate; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide.

perature the preparations were centrifuged for 10 minutes at 2000 × *g* in a small, angle head centrifuge in the cold room. This sedimented the larger cellular débris and floated a mat of fibrous connective tissue. The sediment and connective tissue mass were discarded, and the remaining, cloudy, free flowing fluid, the "partially purified" homogenate, was used in the experiments. In some experiments the homogenate was centrifuged in a Spinco model L preparative ultracentrifuge at 5000 × *g* for 15 minutes to sediment "mitochondria," then at 57,000 × *g* for 60 minutes to sediment "microsomes" and yield a supernatant or "soluble protein" fraction ($S_{57,000}$).

Incubation Methods and Media—The homogenates or cell fractions were incubated in conventional Warburg vessels at 37° with 100 per cent oxygen as the gas phase and alkali in the center wells. The incubation period was 1 hour unless otherwise specified. Each vessel contained 1.0 ml. of homogenate or cell fraction and 2.0 ml. of a mixture containing 30 μmoles of K^+, 10 μmoles of Mg^{++}, 20 μmoles of Cl^-, and 20 μmoles of phosphate buffered at pH 6.9. Where indicated in the text, the incubation mixtures were fortified with 0.01 μmole of cytochrome *c*, 2.5 μmoles of adenosine triphosphate, and 0.75 μmole of diphosphopyridine nucleotide per vessel. Substrates were added at a level of 3.0 or 30.0 μmoles per vessel. The nitrogen content of each homogenate or cell fraction was determined and metabolic rates were calculated in terms of the nitrogen content of the preparation.

In all experiments, unless otherwise noted, estradiol was added to alternate vessels as an aqueous suspension to give a final concentration of 4×10^{-6} M.

Analytical Methods—The incubation medium was analyzed before and after incubation for citric acid by Elliott's modification[2] of the Natelson, Pincus, and Lugovoy method (5). The method of Barker and Summerson (6) was used for lactic acid analyses. Pyruvic, α-ketoglutaric, and oxalacetic acids were measured by a slight modification of the method of Friedemann and Haugen (7).

Results

In experiments reported previously (4), with substrates at a level of 10 μmoles per ml., estradiol was found to increase the utilization of citrate and oxygen and the production of α-ketoglutarate about 30 per cent. When the amount of substrate was decreased to 1 μmole per ml., the system was more sensitive to estradiol and the addition of the hormone increased the utilization of citrate and oxygen and the production of α-ketoglutarate by 50 per cent (Table I). It had been found previously (4) that when no

[2] Personal communication from Dr. K. A. C. Elliott.

citrate was added to the system the addition of estradiol produced only an 11 per cent increase in the utilization of oxygen.

In one series of experiments, the estradiol was tipped into the reaction chamber of the Warburg vessel from the side arm at (1) zero time, (2) after 30 minutes of incubation, or (3) at the end of the 60 minute incubation period. Estradiol had no effect if added at the end of the incubation period. When added 30 minutes before the end of the incubation period, estradiol increased the utilization of citrate and the production of α-ketoglutarate to about 30 per cent over the control values. When estradiol was added at zero time, immediately after the initial observation of the manometers, the utilization of oxygen and citrate and the production of α-ketoglutarate proceeded at rates twice as great as those in the control vessels to which no estradiol was added.

TABLE I

Effect of Estradiol on Citrate Metabolism by Partially Purified Placental Homogenates

The figures are the means of sixteen experiments ± the standard error of the mean.

Citrate concentration, *mmoles per l.* / Estradiol	10 Absent	10 Present	1 Absent	1 Present
Oxygen consumption, *μl. O_2 per hr. per mg. N*	22.24 ±1.35	27.06 ±1.80	15.36 ±0.61	22.46 ±0.80
Citrate utilization, *μmoles per hr. per mg. N*	1.83 ±0.28	2.38 ±0.40	0.44 ±0.022	0.69 ±0.018
α-Ketoglutarate production, *μmoles per hr. per mg. N*	1.20 ±0.11	1.66 ±0.13	0.66 ±0.048	0.97 ±0.053

To determine how quickly the effect of estradiol becomes evident, placental homogenates were incubated with 1 μmole per ml. of citrate, with and without added estradiol; vessels were removed for analysis after 15, 30, 60, and 120 minutes of incubation. The graphs of the utilization of citrate *versus* time (Fig. 1) and the production of α-ketoglutarate *versus* time (Fig. 2) show that the effects of estradiol on metabolism are evident after 30 minutes. In other experiments the effect of estradiol could be detected after as little as 15 minutes of incubation.

An investigation was made of the amount of hormone necessary to produce these effects. Serial dilutions of the stock solution of estradiol were made so that the final concentration of the hormone in the incubation vessel was 1, 0.1, 0.01, or 0.001 γ per ml. These are concentrations of 4×10^{-6}, 4×10^{-7}, 4×10^{-8}, and 4×10^{-9} M respectively. With placental homogenates, or with the fraction not sedimented by 57,000 × *g* for 60 minutes ($S_{57,000}$), the effects of estradiol on the utilization of citrate and on

the production of α-ketoglutarate were evident at the three higher levels, but were not clearly defined at 0.001 γ per ml. (Table II).

In previous experiments in which placental homogenates and higher levels of substrate were used, no effect of progesterone or stilbestrol was detected (4). After the development of this more sensitive reaction system, other steroids were reinvestigated for possible effects on metabolism (Table III). Estriol, progesterone, and stilbestrol were found to have effects similar to

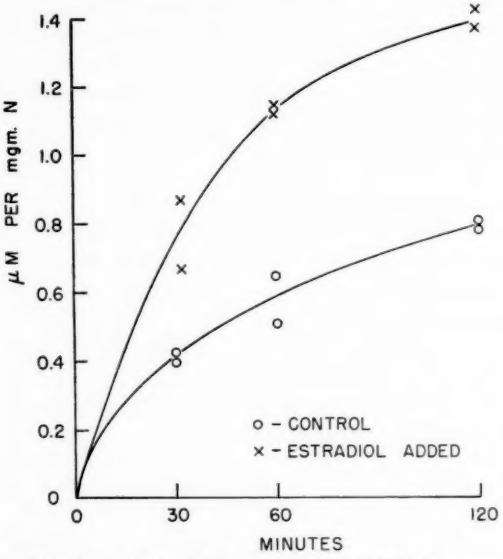


FIG. 1. The utilization of citrate by a partially purified homogenate of human placenta, expressed as micromoles of citrate utilized per mg. of nitrogen in the homogenate, as a function of the length of the incubation period.

that of estradiol, but these effects could be detected only at levels of 10 γ per ml. These substances thus show about one-thousandth as much activity as estradiol. They can be ranked in order of activity in this system as progesterone > stilbestrol > estriol. Progesterone and estradiol together are no more effective than estradiol alone. Testosterone at a level of 17 γ per ml. produced a 50 per cent increase in the utilization of citrate and oxygen and in the production of α-ketoglutarate. When present in lower concentration, it was without effect. Estrone, in contrast, was as effective as estradiol in increasing the metabolism of placental homogenate or supernatant fractions. This particular fraction of human placenta ($S_{57,000}$) contains a very active enzyme catalyzing the reversible conver-

sion of estrone to estradiol,[3] and it is possible that only one of the two forms is effective in metabolism.

Estrogens have been found to have inhibitory action on rat liver slices and homogenates (8, 9). A series of experiments with both homogenates and $S_{57,000}$ fractions of rat liver and kidney showed that these preparations were not stimulated by the addition of estradiol. The homogenates utilized citrate, and the $S_{57,000}$ fractions utilized citrate and accumulated

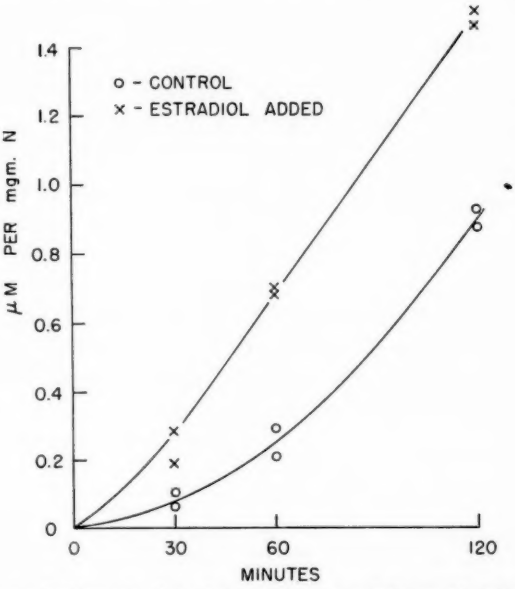


FIG. 2. The production of α-ketoglutarate by a partially purified homogenate of human placenta, expressed as micromoles of α-ketoglutarate produced per mg. of nitrogen in the homogenate, as a function of the length of the incubation period.

α-ketoglutarate. Estradiol, at a level of 1 γ per ml., had neither a stimulatory nor an inhibitory effect on these preparations.

Previous experiments had demonstrated that estradiol stimulates the utilization of pyruvate and the conversion of pyruvate to CO_2 by placental homogenates (4). To determine whether these findings were the indirect result of a stimulation of the citrate → α-ketoglutarate steps or of a more direct effect of the hormone on lactic dehydrogenase, placental homogenates were incubated with pyruvate or lactate (1 μmole per ml.) as the substrate (Table IV). With pyruvate as the substrate, estradiol increased the

[3] Personal communication from Dr. Lewis L. Engel.

utilization of pyruvate, the production of lactate, and the consumption of oxygen. With lactate as the substrate, a net increase of both lactate and α-keto acid was observed during the incubation. The addition of estradiol

TABLE II

Effect of Varying Estradiol Concentrations on Placental Metabolism in Vitro

The figures are the means of duplicate determinations, expressed as micromoles per hour per mg. of N.

	Control	Estradiol added, γ per ml.			
		1	0.1	0.01	0.001
Citrate utilization					
Homogenate	0.59	1.14	0.82	0.65	0.63
$S_{57,000}$	0.28	1.29	0.61	0.49	0.44
α-Ketoglutarate production					
Homogenate	0.66	1.13	0.93	1.02	0.97
$S_{57,000}$	0.07	0.84	0.41	0.44	0.06

TABLE III

Effect of Steroids on Metabolism of Citrate by Placenta

The figures for homogenates are the means ± the standard errors of four determinations; for $S_{57,000}$, the means ± the standard errors of ten determinations; expressed as micromoles per hour per mg. of N.

	Citrate utilization	α-Ketoglutarate production
Homogenate		
Control	0.43 ± 0.005	0.38 ± 0.06
Estradiol, 1 γ per ml.	0.80 ± 0.02	0.71 ± 0.05
Estriol, 10 γ per ml.	0.50 ± 0.02	0.49 ± 0.01
Progesterone, 10 γ per ml.	0.70 ± 0.04	0.62 ± 0.05
Stilbestrol, 10 γ per ml.	0.57 ± 0.05	0.49 ± 0.01
Testosterone, 17 γ per ml.	0.64 ± 0.05	0.54 ± 0.04
$S_{57,000}$		
Control	0.38 ± 0.051	0.16 ± 0.033
Estradiol, 1 γ per ml.	0.88 ± 0.023	0.70 ± 0.044
Estrone, 1 γ per ml.	0.88 ± 0.015	0.71 ± 0.051

increased the utilization of oxygen, but did not alter the rates of production of lactate or α-keto acid. The estradiol-induced increase in the production of lactate with pyruvate as the substrate is probably the result of a DPN-linked oxidation-reduction system. The fact that estradiol has little or no effect on the production of pyruvate or lactate with lactate as the substrate indicates that there is no primary effect of estradiol on the lactic dehydrogenase step.

An attempt was made to localize the estradiol-sensitive system within the cell. Placental homogenates in 0.25 M sucrose were separated by differential centrifugation into mitochondria, microsomes, and supernatant

TABLE IV

Effect of Estradiol on Pyruvate and Lactate Metabolism by Placental Homogenates

The figures are the means ± the standard errors of five determinations.

	Pyruvate		Lactate	
	No estradiol	Estradiol	No estradiol	Estradiol
Oxygen consumption, *μl. per hr. per mg. N*	12.0 ±1.24	16.4 ±1.25	39.7 ±1.40	44.6 ±0.64
Pyruvate utilization or production, *μmoles per hr. per mg. N*	−0.59 ±0.048	−1.16 ±0.058	+0.48 ±0.07	+0.40 ±0.02
Lactate production, *μmoles per hr. per mg. N*	+0.56 ±0.11	+1.15 ±0.11	+0.39 ±0.05	+0.47 ±0.15

TABLE V

Effect of Estradiol on Citrate Metabolism by Placental Cell Fractions

The figures are the means ± the standard errors. The number of experiments is given in parentheses.

	Oxygen consumption, μl. O_2 per hr. per mg. N		Citrate utilization, μmoles per hr. per mg. N		α-Ketoglutarate production, μmoles per hr. per mg. N	
	No estradiol	Estradiol	No estradiol	Estradiol	No estradiol	Estradiol
Mitochondria (8)	24.7 ±3.2	29.5 ±4.2	0.69 ±0.09	0.70 ±0.07	0.51 ±0.06	0.61 ±0.05
Microsomes (8)	11.0 ±1.6	12.4 ±1.8	0.25 ±0.03	0.26 ±0.02	0.29 ±0.04	0.33 ±0.02
$S_{57,000}$ (10)	0	0	0.59 ±0.04	0.97 ±0.01	0.52 ±0.04	1.09 ±0.09
Mitochondria + $S_{57,000}$ (2)	39.0	41.7	0.99	1.18	0.79	0.85
Microsomes + $S_{57,000}$ (6)	24.0 ±1.3	23.6 ±1.3	0.47 ±0.04	0.63 ±0.07	0.92 ±0.07	0.91 ±0.07
S_{5000} (2)	33.6	36.8	1.03	1.73	1.05	2.29
Mitochondria + S_{5000} (4)	35.0 ±0.85	42.0 ±2.1	0.67 ±0.05	0.91 ±0.03	0.87 ±0.09	0.99 ±0.09

($S_{57,000}$) fractions. The $S_{57,000}$ fraction is a water-clear liquid having a pink to cherry color; it contains the soluble enzymes of the cell. It does not utilize oxygen (Table V), for the necessary enzymes were removed with the mitochondria. When incubated with citrate (1 μmole per ml.) as the substrate, it utilizes citrate and accumulates α-ketoglutarate.

Added estradiol markedly increases the conversion of citrate to α-ketoglutarate by the $S_{57,000}$ fraction, but has no effect on separated mitochondria or microsomes. These experiments show that estradiol will increase citrate utilization and α-ketoglutarate production in the absence of oxygen utilization. This is further indication that the action of estradiol is localized in one or more of the Krebs cycle enzymes and not in the electron transmitter cycle of enzymes.

Estradiol increased the utilization of citrate and the accumulation of α-ketoglutarate by washed mitochondria under anaerobic but not under aerobic conditions and only in the presence of DPN and not TPN (Table VI). Apparently some of the estradiol-sensitive enzymes of the $S_{57,000}$ fraction (which require DPN) are also present in the mitochondrial fraction, but can show their effects only anaerobically, when the TPN-requiring isocitric dehydrogenase is less active.

TABLE VI

Effect of Estradiol on Citrate Metabolism by Washed Mitochondria from Placenta

The figures are the means ± the standard errors of five determinations, expressed as micromoles per hour per mg. of N.

Gas phase	Coenzyme added	Citrate utilization		α-Ketoglutarate production	
		No estradiol	Estradiol	No estradiol	Estradiol
O_2	DPN	0.95 ± 0.03	0.96 ± 0.03	0.94 ± 0.05	0.99 ± 0.06
"	TPN	1.35 ± 0.06	1.20 ± 0.07	1.30 ± 0.11	1.33 ± 0.12
N_2	DPN	0.65 ± 0.02	1.01 ± 0.08	0.39 ± 0.15	0.48 ± 0.09
"	TPN	0.97 ± 0.05	0.95 ± 0.05	0.79 ± 0.08	0.85 ± 0.08

The finding that estradiol produces clear, reproducible effects on the soluble enzyme fraction of placenta led us to investigate the coenzymes required for the utilization of citrate, for the production of α-ketoglutarate, and for the effect of estradiol on this system.

In the absence of added cofactors, or with the addition of cytochrome *c* or ATP or both, there was only slight conversion of citrate to α-ketoglutarate and no effect of estradiol was evident (Table VII). When DPN was present, citrate was utilized, α-ketoglutarate accumulated, and the addition of estradiol increased both. When TPN was substituted for DPN, citrate was utilized and α-ketoglutarate accumulated, but there was no acceleration of these processes when estradiol was added. Cytochrome *c* and ATP added with the DPN increased both the utilization of citrate and the production of α-ketoglutarate. An effect of estradiol on the system was evident whenever DPN was present.

Neither coenzyme A nor lipoic acid is required for the system which con-

verts citrate to α-ketoglutarate. The addition of either or both had no effect on the utilization of citrate, the production of α-ketoglutarate, or on the magnitude of the increases in these induced by added estradiol. The addition of lipoic acid (10 γ per ml.) to the $S_{57,000}$ fraction of the placenta increased the utilization of pyruvate from 0.12 to 0.30 μmole per mg. of N per hour. The addition of estradiol further increased this rate to 0.85 μmole per mg. of N per hour. When coenzyme A was added to the placental homogenate at a level of 27 γ per ml., the utilization of pyruvate was increased from 0.79 to 1.07 μmoles per mg. of N per hour. The addition of estradiol further increased this rate to 1.66 μmoles per mg. of N

TABLE VII

Cofactors Required for Effect of Estradiol on Citrate Metabolism

The figures are means ± the standard errors of five determinations, expressed as micromoles per hour per mg. of N.

Cofactors added	Citrate utilized		α-Ketoglutarate produced	
	No estradiol	Estradiol	No estradiol	Estradiol
None	0.07 ± 0.02	0.19 ± 0.03	0	0
Cytochrome *c*	0.26 ± 0.05	0.19 ± 0.04	0.19 ± 0.04	0.07 ± 0.03
ATP	0.21 ± 0.04	0.13 ± 0.03	0.17 ± 0.05	0.07 ± 0.04
DPN	0.39 ± 0.04	0.70 ± 0.03	0.20 ± 0.04	0.65 ± 0.02
TPN	0.48 ± 0.10	0.48 ± 0.10	0.40 ± 0.12	0.50 ± 0.13
Cytochrome *c* + DPN	0.43 ± 0.06	0.84 ± 0.08	0.35 ± 0.07	0.80 ± 0.08
" " + TPN	0.64 ± 0.03	0.65 ± 0.03	0.60 ± 0.04	0.61 ± 0.03
" " + ATP + DPN	0.55 ± 0.13	0.97 ± 0.14	0.56 ± 0.04	1.08 ± 0.16
Cytochrome *c* + ATP + TPN	0.61 ± 0.03	0.61 ± 0.04	0.63 ± 0.03	0.52 ± 0.01

per hour. Each figure is the mean of four experiments. Thus, the presence of lipoic acid or coenzyme A did not eliminate the stimulatory effect of estradiol on the utilization of pyruvate. The effects of the addition of coenzyme A and lipoic acid in this system are comparable to those observed in other tissues (10, 11).

The $S_{57,000}$ fraction retains its activity after storage at 5° for 2 or 3 days, but not after a week. It can be lyophilized and stored as a dry powder for a month or more and show activity when redissolved in buffered saline. The enzymes involved are precipitated by 50 per cent, but not by 20 per cent, saturated ammonium sulfate.

To obtain further evidence of the site of action of estradiol, the incubation media were analyzed for organic acids by silica gel chromatography. Twelve vessels containing homogenate, citrate, cytochrome *c*, DPN, and

ATP without estradiol, and twelve vessels with estradiol, were incubated 1 hour. The contents of each set of twelve vessels were pooled, and aliquots were taken for colorimetric determinations of citrate, α-ketoglutarate, and lactate. The volume of the remainder was measured, and 4 volumes of 95 per ethanol were added to precipitate the proteins and phosphate. The precipitate was removed by centrifugation, and the supernatant fluid was taken to dryness in a stream of dry air at room temperature. It was then taken up in silica gel and H_2SO_4 and placed on a silica gel column.

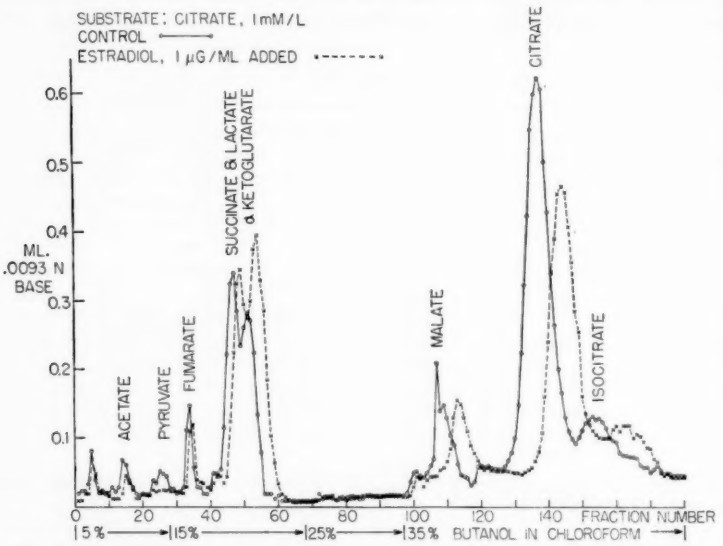


FIG. 3. Chromatograms of the effluent fractions obtained by silica gel chromatography of the incubation media of placental homogenates incubated with citrate as the substrate and with (broken line) and without (solid line) added estradiol.

The organic acids were eluted by butanol-chloroform mixtures containing 5, 15, 25, and 35 per cent *n*-butanol, according to the procedure of Bulen, Varner, and Burrell (12). Chromatograms of placental homogenates incubated 1 hour with citrate as the substrate, with and without estradiol, are presented in Fig. 3. The increased utilization of citrate in the presence of estradiol is evident from the decreased height of the peak compared to the control. The chromatograms show clearly that it is α-ketoglutarate and not some other α-keto acid that is accumulating and that estradiol increases the amount of α-ketoglutarate produced.

The amounts of each of the acids present in the medium at the end of the incubation period were calculated from the chromatograms and are re-

ported in Table VIII. Estradiol increased the utilization of pyruvate, lactate, acetate, and citrate and the accumulation of α-ketoglutarate, but was without effect on fumarate, succinate, and malate. These results confirm our previous observations (4) that the addition of estradiol does not increase the oxygen consumption of placental homogenates incubated with succinate, malate, or oxalacetate as substrate.

TABLE VIII

Organic Acids Present after Incubation of Placental Homogenates with 30 μmoles of Citrate

The figures represent micromoles per 30 ml. of incubation medium.

	Control	Estradiol added	Difference
Acetate	1.42	0.39	−1.03
Pyruvate	1.37	0.30	−1.07
Fumarate	1.40	1.15	−0.25
Lactate	2.48	0.93	−1.55
α-Ketoglutarate	9.94	14.39	+4.45
Succinate	1.29	1.29	0
Malate	3.91	3.73	−0.18
Citrate	14.41	10.09	−4.32
Isocitrate	1.00	0.57	−0.43

DISCUSSION

These experiments show that the metabolism of citrate to α-ketoglutarate by homogenates or cell-free enzyme systems of placenta is stimulated by estradiol, by estrone, and, to a much lesser extent, by estriol, progesterone, stilbestrol, and testosterone. This estradiol-sensitive citrate → α-ketoglutarate system requires DPN and not TPN. In the presence of DPN it will operate anaerobically as well as aerobically. What appears to be a DPN isocitric dehydrogenase in placenta is not unique for vertebrate tissues, for Plaut and Sung (13) found a DPN isocitric dehydrogenase in guinea pig heart, beef heart, and pigeon breast muscle. Whether estradiol stimulates this isocitric dehydrogenase, the conversion of citrate to isocitrate by aconitase, or perhaps both processes, cannot be decided at present. The fact that DPN is required for an acceleration of metabolism by estradiol suggests that the primary effect of the estrogen is a stimulation of the DPN isocitric dehydrogenase. The present experiments provide an example of a clear-cut effect of a hormone on metabolism *in vitro*. An effect of estrogen at this point, by increasing the rate of the Krebs cycle, would increase the rate at which energy is made available to the tissue. This could account for the known physiologic effects of estrogen, such as

the stimulation of the growth of the endometrium and the acceleration of protein synthesis observed *in vivo* (14).

The finding that endometrium (15) and placenta (4) have enzyme systems which respond to estradiol added *in vitro*, whereas the comparable enzymes of liver and kidney do not, is of interest. Liver and kidney do not give gross or histologic evidence of response to estrogens as do endometrium and placenta, two of the normal "target organs" of these hormones.

I want to acknowledge with thanks the cooperation of the staff of the Boston Lying-In Hospital in making these studies possible. My special thanks are due to Janet Loring and Frederica Wellington for their assistance with the experiments and to Dr. John M. Foster and Dr. Edwin E. Gordon for their help with the chromatographic experiments.

SUMMARY

1. The metabolism of citrate to α-ketoglutarate by homogenates or cell-free enzyme systems of placenta, but not of liver or kidney, is stimulated by estradiol, by estrone, and to a much lesser extent, by estriol, progesterone, diethyl stilbestrol, and testosterone.

2. The estradiol-sensitive citrate → α-ketoglutarate system requires DPN and not TPN, and in the presence of DPN will operate anaerobically as well as aerobically.

3. The present evidence suggests that a DPN-linked isocitric dehydrogenase, found primarily in the supernatant (non-particulate) fraction of placental homogenates, is sensitive to stimulation by estradiol.

BIBLIOGRAPHY

1. Hagerman, D. D., and Villee, C. A., *Arch. Biochem. and Biophys.*, **40,** 481 (1952).
2. Villee, C. A., Hagerman, D. D., LaPlace, R., and Quarles, B., *Federation Proc.*, **12,** 284 (1953).
3. Hagerman, D. D., and Villee, C. A., *Endocrinology*, **53,** 667 (1953).
4. Villee, C. A., and Hagerman, D. D., *J. Biol. Chem.*, **205,** 873 (1953).
5. Natelson, S., Pincus, J. B., and Lugovoy, J. K., *J. Biol. Chem.*, **175,** 745 (1948).
6. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138,** 535 (1941).
7. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943).
8. Hayano, M., Schiller, S., and Dorfman, R. I., *Endocrinology*, **46,** 387 (1950).
9. Guidry, M. A., Segaloff, A., and Altschul, A. A., *Endocrinology*, **50,** 29 (1952).
10. Schweet, R. S., and Cheslock, K., *J. Biol. Chem.*, **199,** 749 (1952).
11. Hager, L. P., Fortney, J. D., and Gunsalus, I. C., *Federation Proc.*, **12,** 213 (1953).
12. Bulen, W. A., Varner, J. E., and Burrell, R. C., *Anal. Chem.*, **24,** 187 (1952).
13. Plaut, G. W. E., and Sung, S.-C., *J. Biol. Chem.*, **207,** 305 (1954).
14. Mueller, G. C., and Herranen, A., *Federation Proc.*, **12,** 249 (1953).
15. Hagerman, D. D., and Villee, C. A., *J. Biol. Chem.*, **203,** 425 (1953).

THE PREPARATION AND PROPERTIES OF 3′-TRIPHOSPHOPYRIDINE NUCLEOTIDE*

By LOUIS SHUSTER† and NATHAN O. KAPLAN

(From the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland)

(Received for publication, November 8, 1954)

The coenzyme triphosphopyridine nucleotide (TPN) contains a monoester phosphate group on the 2′ position of the adenylic acid ribose (1). With the aid of a specific 3′-nucleotidase it has been possible to show that the monoester phosphate group on the adenylic acid moiety of coenzyme A is in the 3′ position (2). The monoester phosphate of TPN is not hydrolyzed by 3′-nucleotidase.

Conditions which are known to interconvert the 2′ and 3′ isomers of various nucleotides (3) have been used to produce the 3′ isomer of TPN from the naturally occurring 2′ isomer. Such changes can be detected by the use of 3′-nucleotidase. This report describes the isolation of 3′-TPN and its activity in a number of enzyme systems compared with 2′-TPN.

Materials and Methods

Triphosphopyridine nucleotide of approximately 80 per cent purity was obtained from the Sigma Chemical Company. Another preparation, 92 per cent pure, was made from diphosphopyridine nucleotide (DPN) by means of the pigeon liver DPN-kinase system of Wang, Kaplan, and Stolzenbach (4). The 2′ and 3′ isomers of yeast adenylic acid (corresponding to isomers *a* and *b* respectively) and oxidized glutathione were purchased from the Schwarz Laboratories, Inc.

The 3′-nucleotidase used in these experiments was purified 15- to 25-fold from barley (5) or from rye-grass (6). Crystalline yeast alcohol dehydrogenase was prepared according to Racker (7); crystalline liver alcohol dehydrogenase, according to Bonnichsen and Wassen (8); isocitric dehydrogenase, according to Grafflin and Ochoa (9); crystalline lactic dehydrogenase from pig heart by the method of Straub (10); glutathione reductase from peas by the method of Kaplan, Colowick, and Neufeld (11); pyridine nucleotide transhydrogenase from *Pseudomonas fluorescens* ac-

* Contribution No. 95 of the McCollum-Pratt Institute. Aided by grants from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council, the Rockefeller Foundation, and the Williams Waterman Fund.

† Lalor Predoctoral Fellow. Present address, National Institute for Medical Research, Mill Hill, London, England.

cording to Colowick *et al.* (12); diphosphopyridine nucleotidase from *Neurospora* by the method of Kaplan *et al.* (13); and Taka-Diastase deaminase according to Kaplan, Colowick, and Ciotti (14). The pigeon liver preparation of Wang and Kaplan (15) was used as a source of glutamic dehydrogenase. Glucose-6-phosphate dehydrogenase, prepared from yeast by the method of Kornberg (16), was furnished by Dr. T. P. Wang. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (17) was kindly supplied by Dr. R. D. DeMoss; barium-6-phosphogluconate, 6-phosphogluconic dehydrogenase, and TPN-cytochrome *c* reductase from hog liver (18), by Dr. B. L. Horecker; and a 20-fold purified preparation of nitrate reductase from *Neurospora* (19), by Dr. D. J. D. Nicholas.

The cyanide method of Colowick, Kaplan, and Ciotti (20) was used to determine the concentration of nicotinamide-ribose linkages. Inorganic phosphate was measured by the method of Fiske and Subbarow (21).

Results

Measurement of Nucleotide Isomerization by Means of 3′-Nucleotidase—80 μmoles each of 2′-adenylic acid and 3′-adenylic acid were allowed to stand at room temperature in 4.0 ml. of 0.5 N HCl. At various times 0.1 ml. aliquots were removed, neutralized with 0.05 ml. of 1.0 N NaOH, and the amount of 3′-adenylic acid present was determined by measuring the inorganic phosphate released upon treatment with 3′-nucleotidase. Control samples were also tested in each case for inorganic phosphate. The results of this experiment (Fig. 1) indicate that equilibrium was attained in about 25 hours. The maximal amount of hydrolysis observed under these conditions was less than 5 per cent. Attempts to shorten the time of isomerization by the use of higher temperatures gave considerably more hydrolysis and were therefore abandoned.

This experiment served to establish the feasibility of measuring the interconversion of 2′- and 3′-nucleotides by means of 3′-nucleotidase. The method can be used in either direction and is suitable for kinetic studies. Loring *et al.* (22) have followed the interconversion of the 2′ and 3′ isomers of cytidylic acid by observing changes in optical rotation. Such a method requires pure and concentrated solutions, which is not the case when 3′-nucleotidase is used. Other workers have separated the two isomers by paper chromatography, but this method is more tedious and not so well suited for kinetic work, nor can it be used when the two isomers are not readily separable on paper.

Isomerization of TPN—TPN was treated under the same conditions which were found suitable for interconverting 2′- and 3′-adenylic acid. Treatment of TPN with 0.5 N HCl for 24 hours at room temperature causes very little hydrolysis of either the monoester phosphate or the

nicotinamide-ribose linkage. Under these conditions over 40 per cent of the TPN is converted to a form which no longer reacts with isocitric dehydrogenase. The product of this conversion, unlike the starting material, is split by 3′-nucleotidase into almost equal amounts of DPN and inorganic phosphate. Thus it would appear that the isomerization of TPN has taken place. These results also show that the isomeric form of TPN reacts neither with isocitric dehydrogenase, which is specific for 2′-TPN, nor with yeast alcohol dehydrogenase, which is specific for DPN.

Isolation of 3′-TPN—Attempts to separate the two isomers of TPN by paper chromatography, with the isoamyl alcohol-Na_2HPO_4 system of

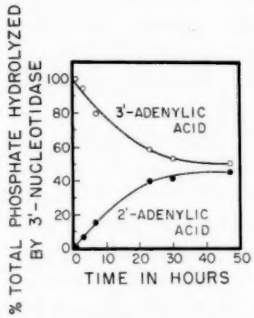


FIG. 1. Isomerization of 2′- and 3′-adenylic acids by 0.5 N HCl. 80 μmoles of adenylic acid were allowed to stand at room temperature in 4.0 ml. of 0.5 N HCl. At various times 0.1 ml. aliquots were removed, neutralized with 0.05 ml. of 1.0 N NaOH, and incubated for 40 minutes at 37° with 1.8 ml. of 0.1 M Tris buffer, pH 7.5, and 0.1 ml. of 3′-nucleotidase. The values are corrected for controls measured without enzyme.

Carter (23) and the pyridine-water system of Burton and San Pietro (24), were unsuccessful. Neither was resolution achieved by chromatography on Dowex 1 resin in the formate form. An attempt was therefore made to destroy selectively the 2′ isomer. This was accomplished by reducing the 2′ isomer with isocitric dehydrogenase and then destroying the 2′-TPNH by acid treatment, leaving the oxidized 3′ isomer intact.

TPN (100 mg.) prepared from DPN by the method of Wang, Kaplan, and Stolzenbach (4) was allowed to stand in 10 ml. of 0.5 N HCl for 28 hours at room temperature. The solution was then neutralized with 1.2 ml. of 5 N KOH. A comparison of the cyanide assay and measurement with isocitric dehydrogenase indicated 49 per cent isomerization of the original TPN. 1.0 ml. of 1.0 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5), 0.2 ml. of 1.0 M $MgCl_2$, 1.0 ml. of 0.1 M sodium isocitrate, and 0.5 ml. of isocitric dehydrogenase were added to the TPN solution, and

the mixture was incubated at 37°. Reduction of the 2′ isomer, as indicated by increase in optical density at 340 mμ, was essentially complete after 10 minutes incubation. At the end of 15 minutes the enzyme was inactivated by heating for 3 minutes at 100°. The solution was then cooled, and a flocculent precipitate of denatured protein was removed by centrifugation. The supernatant solution was made royal blue to Congo red paper with 0.6 ml. of 4 N HNO_3. This destroyed the 2′-TPNH, as indicated by decrease in optical density at 340 mμ. Testing with isocitric dehydrogenase showed that there was no longer any appreciable amount of oxidized 2′-TPN present.

The 3′-TPN and some of the acid degradation products of 2′-TPNH (presumably a nicotinamide product and phosphoadenosine diphosphate ribose, as indicated by the work of Burton and Kaplan (25) with DPN) were precipitated with 5 volumes of cold acetone. The precipitate was air-dried and dissolved in 11 ml. of H_2O. Some insoluble material, apparently denatured protein, was removed by centrifugation. The solution was then brought to pH 8.5 with 0.2 ml. of 5 N KOH and put on a column of Dowex 1 resin in the formate form, 2 cm. in diameter by 1.5 cm. high. After the column was washed with 50 ml. of H_2O, the 3′-TPN was eluted with a solution which was 0.1 M with respect to both formic acid and sodium formate. The progress of this elution was followed by measuring the optical density at 260 mμ of 0.1 ml. aliquots in 3 ml. of H_2O. Peak fractions were pooled, and the combined solution of 60 ml. was made royal blue to Congo red paper with 2.0 ml. of 4 N HNO_3. The 3′-TPN was then precipitated with 4 volumes of cold acetone.

The acetone precipitate was centrifuged, dissolved in 5 ml. of H_2O, reprecipitated from acid solution with 25 ml. of cold acetone, and washed with 40 ml. of cold acetone. The precipitate was then air-dried and dissolved in 5 ml. of H_2O, and the pH of the solution was brought to neutrality with 0.1 ml. of 0.1 N NaOH.

Chromatography was effective in removing the adenine-containing split-product of 2′-TPNH, since this resulted in a decrease of the $D_{260}:D_{325}$ ratio in 1.0 M KCN from 5.34 to 2.59. The ratio calculated for oxidized TPN is 2.62. The 3′-TPN preparation was also free of isocitrate when tested with 2′-TPN and isocitric dehydrogenase.

The yield of 3′-TPN obtained in this case was rather low, only 10.2 μmoles out of a calculated possible total of 50 μmoles. An analysis of this preparation is presented in Table I.

Preparation of Reduced TPN—12 mg. of a dry isomeric mixture of approximately equal parts of 2′-TPN and 3′-TPN were placed in a Thunberg tube together with 25 mg. of $NaHCO_3$ and 15 mg. of sodium hydrosulfite. The tube was evacuated, and 2.5 ml. of H_2O were tipped in from the side

arm. After incubation for 25 minutes at 37° the tube was chilled, air was admitted, and 0.5 ml. of 0.1 N NaOH was added. The tube was then warmed to room temperature, and oxygen was bubbled through the solution for 5 minutes. The yield, as determined from optical density at 340 mμ, was 8.3 μmoles of reduced pyridine nucleotide. The same amount of 2′-TPN was also reduced in this manner to serve as a control.

The reduction of isolated 3′-TPN was carried out in a similar fashion. 1.2 ml. of a solution containing 2 μmoles of 3′-TPN and 200 μmoles of $NaHCO_3$ were tipped from the side arm into an evacuated Thunberg tube containing 10 mg. of sodium hydrosulfite. The tube was incubated for 10 minutes at 37°, then chilled. The vacuum was released, 0.1 ml. of 1.0 N KOH was added, and oxygen was bubbled through the solution for 5 minutes. A similar amount of 2′-TPN was reduced in the same manner to serve as a control.

TABLE I

Analysis of 3′-TPN

For methods see the text.

	μmole
Nicotinamide-ribose	0.22
2′-TPN	0.01
DPN	0
PO_4 after 3′-nucleotidase	0.20
DPN after 3′-nucleotidase	0.185

Comparison of 3′-TPN with 2′-TPN in Some Dehydrogenase Systems—Some of the tests described here were carried out with an isomeric mixture of approximately equal amounts of 2′-TPN and 3′-TPN, while for the others a preparation of isolated 3′-TPN was used. The presence of either form of TPN apparently does not affect the reactions of the other isomer.

Isocitric Dehydrogenase from Pig Heart—This system was shown by Adler *et al.* (26) to work with TPN but not with DPN. Fig. 2, *a* shows that 3′-TPN will not react in this system, since only about one-half of the isomeric mixture of 2′-TPN and 3′-TPN becomes reduced with this enzyme.

Yeast Glucose-6-phosphate Dehydrogenase—Warburg *et al.* (27) found that this system requires TPN and will not work with DPN. As indicated in Fig. 2, *b*, this enzyme fails to catalyze the reduction of 3′-TPN contained in an isomeric mixture.

6-Phosphogluconic Dehydrogenase—The TPN specificity of this system was established by Warburg and Christian (28). This is another enzyme which fails to reduce the 3′-TPN present in an isomeric mixture (Fig. 2, *d*).

Liver Alcohol Dehydrogenase—Unlike the yeast alcohol dehydrogenase,

this enzyme will reduce TPN, although at a much slower rate than DPN (29). Fig. 2, *c* shows that 3′-TPN is reduced by this system at the same rate that 2′-TPN is.

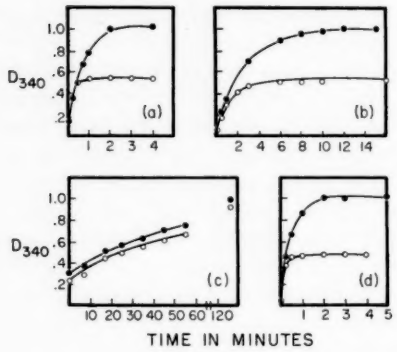


FIG. 2

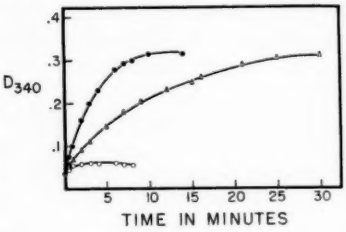


FIG. 3

FIG. 2. Time curves for comparison of 3′-TPN with 2′-TPN in some dehydrogenase systems. ●, 2′-TPN; ○, isomeric mixture of 2′-TPN and 3′-TPN. The reaction mixture (3.0 ml.) in each case contained 0.45 μmole of 2′-TPN or 0.42 μmole of isomerized TPN. Additional components are described below. (*a*) Isocitric dehydrogenase from pig heart. Additions, 2.8 ml. of 0.1 M Tris buffer, pH 7.5, 0.1 ml. of enzyme, and 0.02 ml. of 0.1 M sodium isocitrate to start the reaction. (*b*) Yeast glucose-6-phosphate dehydrogenase. Besides TPN the reaction mixture contained 0.1 ml. of 0.1 M glucose-6-phosphate, 2.8 ml. of 0.1 M Tris buffer, pH 7.5, and 0.1 ml. of enzyme. (*c*) Liver alcohol dehydrogenase. The reaction in 3.0 ml. of 0.1 M alcohol-Tris mixture was started by the addition of 0.3 ml. of enzyme. (*d*) 6-Phosphogluconic dehydrogenase. Additions other than TPN were 0.1 ml. of 0.1 M $MgCl_2$, 2.7 ml. of 0.1 M Tris, pH 7.5, and 0.1 ml. of enzyme. The reaction was started by the addition of 0.1 ml. of 0.05 M potassium-6-phosphogluconate.

FIG. 3. Time curves for comparison of 3′-TPN with 2′-TPN in the glucose-6-phosphate dehydrogenase system of *L. mesenteroides*. ●, 2′-TPN; ○, 3′-TPN; △, 3′-TPN after treatment with 3′-nucleotidase. The reaction mixture (3.0 ml.) contained 0.2 μmole of 2′-TPN or 3′-TPN, 0.1 ml. of 0.1 M glucose-6-phosphate, and 2.8 ml. of 0.1 M Tris buffer, pH 7.5. The reaction was started with 0.05 ml. of enzyme. To show that failure of 3′-TPN to react in this system was not due to inhibition or inactivation of the enzyme, 0.2 μmole of 3′-TPN was incubated with 0.1 ml. of 3′-nucleotidase and 0.3 ml. of 0.1 M Tris buffer, pH 7.5, for 17 minutes at 37°. Then 2.3 ml. of 0.1 M Tris buffer, pH 7.5, and 0.1 ml. of 0.1 M glucose-6-phosphate were added and the reaction was started with 0.05 ml. of enzyme.

Glucose-6-phosphate Dehydrogenase from L. mesenteroides—The failure of this enzyme, which can reduce both DPN and 2′-TPN, to reduce 3′-TPN is illustrated in Fig. 3. This failure is not due to inhibition or inactivation of the enzyme, since there is no interference by 3′-TPN with the reduction of added 2′-TPN. The dephosphorylation of 3′-TPN by 3′-nucleotidase

to DPN allows the reaction to proceed at the rate usually obtained with DPN. A possible explanation might be that this preparation contains two enzymes, one specific for 2′-TPN and the other for DPN.

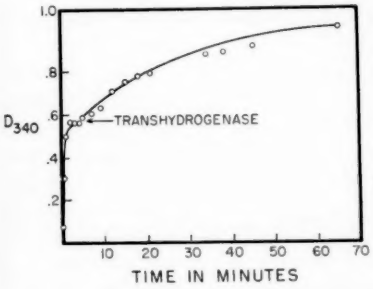


FIG. 4

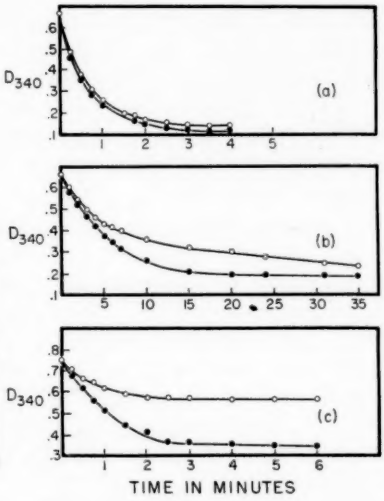


FIG. 5

FIG. 4. The reduction of 3′-TPN by 2′-TPNH and pyridine nucleotide transhydrogenase. The reaction mixture (3.0 ml.) contained 0.42 μmole of isomerized TPN, 2.8 ml. of 0.1 M phosphate buffer, pH 7.5, and 0.05 ml. of isocitric dehydrogenase. The reaction was started with 0.1 ml. of 0.05 M sodium isocitrate. When the reduction of the 2′-TPN present in the mixture was complete, 0.1 ml. of purified *Pseudomonas* transhydrogenase was added.

FIG. 5. Time curves for comparison of 3′-TPNH with 2′-TPNH in some electron transport systems. ●, 2′-TPNH; ○, isomeric mixture of 2′-TPNH and 3′-TPNH. The reaction mixture (3.0 ml.) in each case contained 0.1 ml. of 2′-TPNH or isomerized TPNH and the additions noted below. (*a*) Lactic dehydrogenase. Additions other than TPNH were 0.1 ml. of 0.1 M sodium pyruvate and 2.8 ml. of 0.1 M phosphate buffer, pH 7.5. The reaction was started with 0.05 ml. of enzyme. (*b*) Glutamic dehydrogenase. Besides TPNH the reaction mixture contained 0.1 ml. of 1.0 M NH_4Cl, 2.6 ml. of 0.1 M phosphate buffer, pH 7.5, and 0.1 ml. of enzyme. The reaction was started by the addition of 0.1 ml. of 0.1 M sodium α-ketoglutarate. (*c*) Glutathione reductase. Additions, 0.1 ml. of 0.5 M oxidized glutathione and 2.7 ml. of 0.1 M phosphate buffer, pH 7.5. The reaction was started with 0.1 ml. of enzyme.

Pyridine Nucleotide Transhydrogenase—This enzyme is relatively unspecific and will catalyze the transfer of electrons between a number of oxidized and reduced pyridine nucleotides (30). As shown in Fig. 4, it will also catalyze the reduction of 3′-TPN by 2′-TPNH generated with isocitric

dehydrogenase. At present, the use of the transhydrogenase system is the most convenient method for generating enzymatically reduced 3′-TPNH.

Comparison of 3′-TPNH with 2′-TPNH in Some Pyridine Nucleotide Systems; Lactic Dehydrogenase—Mehler *et al.* (31) have found that TPN functions in this system at a rate which is less than 1 per cent of that obtained with DPN. Fig. 5, *a* shows that the 3′-TPNH present in an isomeric mixture is oxidized by this enzyme at the same rate that 2′-TPNH is.

Glutamic Dehydrogenase—This enzyme can oxidize both TPNH and DPNH at approximately equal rates (32). 3′-TPNH in an isomeric mixture is also oxidized by this enzyme, but at a somewhat slower rate than is 2′-TPNH (Fig. 5, *b*).

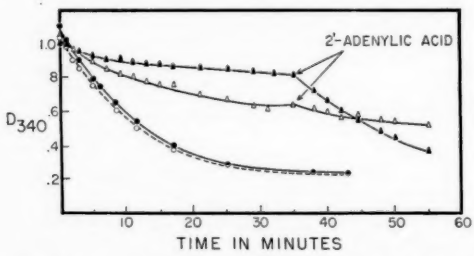


FIG. 6. Effect of phosphate and 2′-adenylic acid on the reduction of DPN by *Pseudomonas* pyridine nucleotide transhydrogenase and the two isomers of TPNH. The reaction mixture contained 0.17 μmole of 2′-TPNH or 3′-TPNH, 0.23 μmole of DPN, 0.1 ml. of yeast alcohol dehydrogenase, 10 μmoles of acetaldehyde, 0.2 ml. of 0.1 M Tris or potassium phosphate buffer, pH 7.5, and water to a volume of 1.0 ml. The reaction was started with 0.05 ml. of transhydrogenase, and 4 μmoles of 2′-adenylic acid were added at the time indicated. ▲, 3′-TPNH, PO_4; △, 2′-TPNH, PO_4; ○, 3′-TPNH, Tris; ●, 2′-TPNH, Tris.

Glutathione Reductase—This is a TPN-specific enzyme (33). Fig. 5, *c* shows that it fails to oxidize the 3′-TPNH present in an isomeric mixture.

TPN-Cytochrome c Reductase—Hog liver contains two cytochrome *c* reductases, one specific for DPN and the other for TPN (18). The TPN-specific enzyme does not catalyze the reduction of cytochrome *c* by 3′-TPNH.

Nitrate Reductase—As shown by Nason and Evans (19), this enzyme, when prepared from *Neurospora crassa*, is specific for TPNH. We have found that 3′-TPNH is completely inactive in this system.

Reduction of DPN by 3′-TPNH and Bacterial Pyridine Nucleotide Transhydrogenase—Both the rate and the extent of the reduction of DPN by TPNH and bacterial pyridine nucleotide transhydrogenase are inhibited by inorganic phosphate. This inhibition can be reversed by the addition of 2′-adenylic acid or its derivatives (30). The 3′ isomer of adenylic acid

cannot replace the 2′ isomer in this reversal. As shown in Fig. 6, both isomers of TPNH are oxidized at the same rate when this reaction is carried out in 0.2 M Tris buffer. In 0.2 M phosphate the oxidation of 3′-TPNH is inhibited more strongly than the oxidation of 2′-TPNH. Under these conditions the addition of 2′-adenylic acid to a final concentration of 4×10^{-3} M is insufficient to cause significant reversal of this inhibition in the case of 2′-TPNH, but will markedly reverse the inhibition of 3′-TPNH oxidation.

Comparison of Hydrolysis of 3′-TPN and 2′-TPN by Neurospora DPNase—Diphosphopyridine nucleotidase from *Neurospora* catalyzes the hydrolysis of the nicotinamide-ribose linkage of both DPN and TPN (13). 3′-TPN is split by this enzyme at a somewhat faster rate than 2′-TPN. Under the conditions used, DPN is split about twice as fast as TPN.

Deamination of 3′-TPN by Taka-Diastase Deaminase—Purified Taka-Diastase deaminase will deaminate the 3′ and 5′ isomers of adenylic acid and DPN, but not the 2′ isomer or TPN (14). This is taken as further evidence that the monoester phosphate of TPN is in the 2′ position. The 3′,5′-diphosphoadenosine fragment obtained from coenzyme A is deaminated at about one-fourth the rate of 3′-adenylic acid, while the corresponding 2′,5′-diphosphoadenosine from 2′-TPN is not affected (2). We have found that 3′-TPN is very slowly deaminated by Taka-Diastase deaminase, whereas the 2′-TPN is not. The rate was somewhat less than 1 per cent of the rate at which 3′-adenylic acid is deaminated under the same conditions.

DISCUSSION

Removal of the monester phosphate group of TPN to give DPN produces marked changes in coenzyme activity. Similar changes have been reported when the monoester phosphate group of coenzyme A is removed to give dephosphocoenzyme A (34). The results of this investigation indicate that shifting the monoester phosphate from the 2′ position on TPN to the 3′ position will also bring about pronounced changes in coenzyme activity.

Preliminary experiments indicate that treatment with 0.5 N HCl for 24 hours at room temperature can bring about a 50 per cent decrease in the amount of inorganic phosphate produced by the action of 3′-nucleotidase on coenzyme A. Should this change be a result of isomerization, it may be possible to separate the resulting 2′-coenzyme A by first dephosphorylating the 3′-coenzyme A in the mixture with 3′-nucleotidase, since coenzyme A and dephosphocoenzyme A exhibit different chromatographic behaviors.

Another case in which such 2′,3′ isomerization might possibly influence biological activity is vitamin B_{12}, in which acid-induced isomerization of the α-ribazole phosphate[1] moiety has been reported, but the exact position

[1] We wish to thank Dr. Karl Folkers for a sample of the ribazole phosphate.

of the monoester phosphate is still unknown (35). This compound is not hydrolyzed by 3′-nucleotidase, even after isomerization.

The results obtained with the enzyme systems tested indicate that both oxidized and reduced 3′-TPN will react with enzymes capable of functioning with either TPN or DPN, but not with enzymes which are strictly specific for one of these coenzymes. A possible exception may be glucose-6-phosphate dehydrogenase from *L. mesenteroides*, which can reduce DPN and 2′-TPN, but not 3′-TPN. Although the activities of this enzyme with both DPN and 2′-TPN run parallel over a 20-fold purification (17), the presence of two separate enzymes of different specificity cannot as yet be ruled out. It is of interest to note that the oxidation of 2-deoxyglucose-6-phosphate by this enzyme will take place with TPN, but not with DPN (R. D. DeMoss, personal communication). If there are two glucose-6-phosphate dehydrogenases in *Leuconostoc*, then tests with 3′-TPN may prove helpful in determining whether a single enzyme can react with both pyridine nucleotides or whether two enzymes are involved.

The observations made with pyridine nucleotide transhydrogenase from *P. fluorescens* can be interpreted in the light of the hypothesis proposed by Kaplan *et al.* (30) to explain the effects of 2′-adenylic acid on this system. They suggest a competition between TPN and 2′-adenylic acid for a site on the enzyme. If the accumulation of TPN on the enzyme inhibits, as in the case of the reduction of DPN by TPNH, this competition would result in apparent activation of the enzyme by 2′-adenylic acid. If the presence of TPN on this site is necessary for the reaction to take place, as in the case of a number of TPN dehydrogenases, 2′-adenylic acid will inhibit (36). In addition, 2′-adenylic acid may apparently bring about direct activation by combining with another site on the enzyme, and it is this site which may be affected by the presence of inorganic phosphate. Such direct activation has been observed, for instance, in the reduction of deamino-DPN by transhydrogenase and DPNH (30). In all cases the 2′ configuration is specifically required for activation, and 3′-adenylic acid will not substitute for the 2′ isomer or its derivatives. For this reason the oxidation of 3′-TPNH by transhydrogenase and DPN may be more strongly inhibited by inorganic phosphate than the oxidation of 2′-TPNH, since there may be activation by the monoester grouping of the 2′-TPNH. On the other hand, the oxidized 2′-TPN formed could hinder the activation of 2′-adenylic acid by competing for the active site of the transhydrogenase, but the 3′-TPN will not, because of the strict 2′ specificity of the site involved.

One way of predicting whether or not a dehydrogenase might react with 3′-TPN could be to test its sensitivity to 2′-adenylic acid. The inhibition of an enzyme such as isocitric dehydrogenase by 2′-adenylic acid, but not

by 3′-adenylic acid (36), indicates a high degree of specificity for the 2′ configuration, and this enzyme would therefore not be expected to function with 3′-TPN.

SUMMARY

1. The acid-catalyzed interconversion of the 2′- and 3′-adenylic acids has been followed in both directions by the use of the specific 3′-nucleotidase.

2. By means of this enzyme it has been possible to demonstrate the acid-catalyzed conversion of the naturally occurring 2′-TPN to the 3′ isomer.

3. The 3′ isomer has been isolated and tested in a number of enzyme systems. In general it will react only in systems which can function with either DPN or 2′-TPN, but not in systems which are absolutely specific for one of these coenzymes. An exceptional case is glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, which can reduce both DPN and 2′-TPN, but fails to react with 3′-TPN.

4. The 3′-TPN is split by *Neurospora* DPNase at a somewhat faster rate than is 2′-TPN. Unlike 2′-TPN, it is slowly deaminated by Taka-Diastase deaminase.

5. The oxidation of 3′-TPNH by bacterial pyridine nucleotide transhydrogenase is inhibited more strongly by inorganic phosphate than that of 2′-TPNH. The addition of 2′-adenylic acid is much more effective in reversing this inhibition with 3′-TPNH than with 2′-TPNH. The significance of these results is discussed with respect to the mechanism of transhydrogenase action.

BIBLIOGRAPHY

1. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **186**, 557 (1950).
2. Wang, T. P., Shuster, L., and Kaplan, N. O., *J. Biol. Chem.*, **206**, 299 (1954).
3. Brown, D. M., and Todd, A. R., *J. Chem. Soc.*, 44 (1952).
4. Wang, T. P., Kaplan, N. O., and Stolzenbach, F. E., *J. Biol. Chem.*, **211**, 465 (1954).
5. Shuster, L., and Kaplan, N. O., *J. Biol. Chem.*, **201**, 535 (1953).
6. Shuster, L., and Kaplan, N. O., in Colowick, S. P., and Kaplan, N. O., Methods in enzymology, New York, **2**, in press.
7. Racker, E., *J. Biol. Chem.*, **184**, 313 (1950).
8. Bonnichsen, R. K., *Acta. chem. Scand.*, **4**, 715 (1950).
9. Grafflin, A. L., and Ochoa, S., *Biochim. et biophys. acta*, **4**, 205 (1950).
10. Straub, F. B., *Biochem. J.*, **34**, 483 (1940).
11. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **205**, 1 (1953).
12. Colowick, S. P., Kaplan, N. O., Neufeld, E. F., and Ciotti, M. M., *J. Biol. Chem.*, **195**, 95 (1952).
13. Kaplan, N. O., Colowick, S. P., and Nason, A., *J. Biol. Chem.*, **191**, 473 (1951).
14. Kaplan, N. O., Colowick, S. P., and Ciotti, M. M., *J. Biol. Chem.*, **194**, 579 (1952).
15. Wang, T. P., and Kaplan, N. O., *J. Biol. Chem.*, **206**, 311 (1954).

16. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
17. DeMoss, R. D., *J. Cell. and Comp. Physiol.*, **41,** suppl. 1, 207 (1953).
18. Horecker, B. L., *J. Biol. Chem.*, **183,** 593 (1950).
19. Nason, A., and Evans, H. J., *J. Biol. Chem.*, **202,** 655 (1953).
20. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., *J. Biol. Chem.*, **191,** 447 (1951).
21. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
22. Loring, H. S., Hammell, M. E., Levy, L. W., and Bortner, H. W., *J. Biol. Chem.*, **196,** 821 (1952).
23. Carter, C. E., *J. Am. Chem. Soc.*, **72,** 1466 (1950).
24. Burton, R. M., and San Pietro, A., *Arch. Biochem. and Biophys.*, **48,** 184 (1954).
25. Burton, R. M., and Kaplan, N. O., *J. Biol. Chem.*, **206,** 283 (1954).
26. Adler, E., von Euler, H., Gunther, G., and Plass, M., *Biochem. J.*, **33,** 1028 (1939).
27. Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282,** 157 (1935).
28. Warburg, O., and Christian, W., *Biochem. Z.*, **292,** 287 (1937).
29. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, **194,** 593 (1952).
30. Kaplan, N. O., Colowick, S. P., Neufeld, E. F., and Ciotti, M. M., *J. Biol. Chem.*, **205,** 17 (1953).
31. Mehler, A., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174,** 961 (1948).
32. von Euler, H., Adler, E., Gunther, G., and Das, N. B., *Z. physiol. Chem.*, **254,** 61 (1938).
33. Mapson, L. W., and Goddard, D. R., *Biochem. J.*, **49,** 592 (1951).
34. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365 (1951).
35. Kaczka, E. A., Heyl, D., Jones, W. H., and Folkers, K., *J. Am. Chem. Soc.*, **74,** 5549 (1952).
36. Neufeld, E. F., Kaplan, N. O., and Colowick, S. P., *Biochim. et biophys. acta*, in press.

THE REACTION OF PYRIDINE NUCLEOTIDES WITH TRIFLUOROACETIC ACID ANHYDRIDE*

By LOUIS SHUSTER,† NATHAN O. KAPLAN, AND FRANCIS E. STOLZENBACH

(From the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland)

(Received for publication, November 8, 1954)

The 3′ isomer of triphosphopyridine nucleotide (TPN) is inactive in a number of enzyme systems which operate with the naturally occurring 2′ isomer (1). Because of this difference an attempt was made to prepare the corresponding 2′,3′ cyclic phosphate, since this type of compound would be related to both isomers.

Brown, Magrath, and Todd (2) have successfully prepared the cyclic 2′,3′-phosphate esters of a number of nucleosides by treating the corresponding 2′- or 3′-mononucleotides with trifluoroacetic acid anhydride (TFAA). The initial reaction in this case is believed to involve the formation of a mixed anhydride of trifluoroacetic acid and nucleoside phosphoric acid. This mixed anhydride then forms the cyclic phosphate ester with the adjacent free hydroxyl of the ribose moiety. One way of picturing this reaction is as an alcoholysis of the mixed anhydride by the adjacent hydroxyl group.

Upon treatment of TPN with trifluoroacetic acid anhydride the expected cyclization could not be detected. Further investigation and similar experiments with diphosphopyridine nucleotide (DPN) showed that what apparently did take place was a selective cleavage of the pyrophosphate linkage, followed by a recombination of part of the resulting fragments to yield several new compounds. One of these compounds obtained by treating DPN with TFAA has been identified as diadenosine-5′-pyrophosphate ($(AR)_2POP$). Another is apparently dinicotinamide-riboside-5′-pyrophosphate ($(NR)_2POP$). The isolation and characterization of these compounds are described in this paper.

Materials and Methods

DPN of 90 per cent purity was obtained from the Pabst Brewing Company. TPN was purchased from the Sigma Chemical Company and was

* Contribution No. 98 of the McCollum-Pratt Institute. Aided in part by grants from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council, the American Trudeau Society Medical Section of the American Tuberculosis Association, and the Rockefeller Foundation.

† Lalor Predoctoral Fellow. Present address, National Institute for Medical Research, Mill Hill, London, England.

approximately 80 per cent pure. Nicotinamide mononucleotide (NMN) was prepared from DPN with snake venom pyrophosphatase. Trifluoroacetic acid anhydride was purchased from the Columbia Organic Chemicals Company, Columbia, South Carolina, and stored at 8°. Because of the toxicity of this compound most of the reactions involving TFAA were carried out in the hood.

The apparatus used for paper electrophoresis was similar to that described by Markham and Smith (3). Inorganic phosphate was determined by the method of Fiske and Subbarow (4). The concentration of nicotinamide-ribose linkages was measured by the cyanide method of Colowick, Kaplan, and Ciotti (5). A modified orcinol method (6) was used for the determination of ribose.

Prostatic phosphatase was prepared according to Markham and Smith (3). Crystalline yeast alcohol dehydrogenase was prepared by the method of Racker (7). The preparation of isocitric dehydrogenase from pig heart was according to Grafflin and Ochoa (8). 5′-Adenylic acid deaminase was prepared from rabbit muscle by the method of Nikiforuk and Colowick (9). Intestinal phosphatase was purchased from the Armour Laboratories. Diphosphopyridine nucleotidase was purified from *Neurospora* by the method of Kaplan, Colowick, and Nason (10). Pyrophosphatase from snake venom was purified according to Astrachan, Wang, and Kaplan.[1] 3′-Nucleotidase was prepared from rye-grass (12).

Results

Reaction of TPN with TFAA—0.5 ml. of TFAA was added to 50 mg. of TPN in a 12 ml. glass centrifuge tube. The tube was stoppered and allowed to stand at room temperature. There was some effervescence upon mixing of the two compounds, and after 45 minutes the TPN was completely in solution. At the end of 12 hours most of the TFAA was removed from the pale brown solution by evaporation under reduced pressure, leaving an amber-colored gum. 5 ml. of ethyl ether were added with stirring, and the resulting suspension was centrifuged, and the residue washed three times with 5 ml. of ether and dried in air. 2 ml. of cold absolute ethanol saturated with NH_3 gas were added to the dry powder thus obtained, and the tube was allowed to stand on ice for 20 minutes. The yellowish precipitate which formed was centrifuged, washed three times with 2 ml. of absolute ethanol, air-dried, and dissolved in 2 ml. of H_2O.

Testing with cyanide showed a recovery of about 80 per cent of the amount of nicotinamide-ribose originally present. No DPN, as measured by yeast alcohol dehydrogenase, could be detected. The solution was

[1] The method is essentially the same as that used by Hurst and Butler (11) for fractionating the snake venom diesterase.

acidified with 0.2 ml. of 4 N HNO_3, and 10 ml. of cold acetone were added. The precipitate which formed was centrifuged, suspended in 2 ml. of H_2O, and any material which failed to dissolve was removed by centrifugation. Subsequently it was observed that some of the reaction products are moderately soluble in both ether and acetone, which would account for the considerable losses entailed in these procedures. The analysis of the final solution is reported in Table I.

It was also observed that deamination of the adenine-containing reaction products by an intestinal phosphatase preparation, as measured by decrease in optical density at 265 mμ, proceeded to completion at the same rate that the deamination of TPN did. Since the deaminase in this preparation is strictly specific for free adenosine, it appeared that bound ade-

TABLE I

Products of Reaction of TPN with TFAA

Compound	Method	
		μmoles
Adenine	D_{260}*	19.4
Nicotinamide-ribose	Cyanide	15.6
DPN	Yeast alcohol dehydrogenase	0
2′-TPN	Isocitric dehydrogenase	1.3
3′-TPN	Yeast alcohol dehydrogenase after 3′-nucleotidase	1.4
Total 3′-phosphate	3′-Nucleotidase	6.2
Cyclic TPN	2′-TPN after acid treatment	0

* To correct for absorption at 260 mμ due to nicotinamide compounds the value of 18.0 was used for the millimolar extinction coefficient.

nosine could be released from the TFAA products by the action of diesterase and monoesterase, which are also present, in the same manner that it is released from TPN.

The presence of cyclic TPN was ruled out by failure to obtain additional 2′-TPN by hydrolysis with 0.1 N HCl for 15 minutes at 100°, these conditions being sufficient to cleave nucleoside cyclic phosphates to an isomeric mixture of 2′- and 3′-nucleotides (13). Treatment with a Taka-Diastase preparation, which readily dephosphorylates cyclic nucleotide phosphates by means of a diesterase and an accompanying specific 3′-nucleotidase, failed to produce more DPN or inorganic phosphate than could be obtained with 3′-nucleotidase alone.[2]

Paper Electrophoresis of Reaction Products—Aliquots of known TPN and the TFAA reaction products were placed on a No. 3 Whatman filter paper

[2] Dr. Leon Heppel has informed us that attempts to form the cyclic TPN by treatment with barium carbonate have also been unsuccessful.

strip moistened with 0.05 M ammonium acetate buffer, pH 3.5. A potential gradient of 20 volts per cm. was applied for 2 hours, and spots were located by examination with a Mineralight ultraviolet lamp. The presence of nicotinamide-ribose linkages was determined by noting the appearance of fluorescence under ultraviolet light after streaking with 1.0 M KCN (14). The mobilities of the spots and their reaction with cyanide (Table II) indicate that, besides TPN, the products include a new, low charged nicotinamide-ribose compound and several new adenine com-

TABLE II

Paper Electrophoresis of Reaction Products of TFAA with DPN and TPN

A potential gradient of 20 volts per cm. was applied for 2 hours in 0.05 M ammonium acetate buffer, pH 3.5. Spots were identified by quenching of fluorescence under ultraviolet light (last column). The appearance of strong ultraviolet fluorescence after streaking with 1.0 M KCN was taken to indicate the presence of nicotinamide-ribose linkages. Mobility is expressed as the distance in cm. from the starting point in the direction of the anode.

Compound	Mobility	Nicotinamide-riboside
TPN	12.0	+
TPN-TFAA products. Band 1	2.0	+
" " " 2	12.0	+
" " " 3	13.5 (Fused)	−
" " " 4	15.0 (Fused)	−
DPN	6.5	+
DPN-TFAA products. Band 1	−1.0	?
" " " 2	1.5	+
" " " 3	3.5	+
" " " 4	6.5	+
" " " 5	10.5	−

pounds which move at a faster rate than TPN and do not contain the nicotinamide-ribose linkage.

Reaction of DPN with TFAA—Christie *et al.* (15) have demonstrated that TFAA can catalyze the condensation of 2 molecules of 5′-adenylic acid to form diadenosine-5′-pyrophosphate. It was felt that the results obtained with TPN might possibly be explained by cleavage of the pyrophosphate linkage, followed by a recombination of the fragments. Since TFAA might also bring about the cyclization and isomerization of the monoester phosphate group of TPN, the range of diadenosine pyrophosphate compounds which could be obtained by a reaction of this type would make analysis of the products rather difficult. Such complications could be avoided by the use of DPN instead of TPN.

XUM

50 mg. of DPN were treated with TFAA, and the products were isolated in the manner described for TPN. As in the case of TPN, only a small amount of unchanged coenzyme (determined with alcohol dehydrogenase) could be detected in the products, although the nicotinamide-ribose linkage appeared to remain largely intact. The results of paper electrophoresis of DPN and the TFAA products are reported in Table II. As in the case of TPN, the main nicotinamide-ribose product appeared to move 1.5 to 2.0 cm. under the conditions used.

Electrophoresis was carried out on a larger scale, the solution being applied as a streak across the width of the paper strip. Bands corresponding to the spots listed in Table II were identified under ultraviolet light and cut out of the paper. One-half of each band was eluted with 3.5 ml. of H_2O, the other half with 3.5 ml. of 1.0 M KCN, and absorption spectra of the eluates were measured in the range 240 to 350 mμ. Only the eluates of Bands 4 and 5 showed the sharp absorption maximum at 260 mμ which is characteristic of adenine compounds. The spectra of the eluates of the remaining three bands had a low broad peak at 260 mμ, such as is typically obtained with nicotinamide compounds. The cyanide eluate of Band 5 failed to absorb appreciably at 325 mμ, indicating the absence of any nicotinamide-ribose linkages. The cyanide eluates of Bands 2 and 4 had well defined spectral peaks at 325 mμ, while the absorption at 325 mμ of cyanide eluates (Bands 1 and 3) was considerably less than the absorption at 260 mμ.

The aqueous eluate of Band 5 was not deaminated by 5′-adenylic acid deaminase, but was deaminated completely by intestinal phosphatase, indicating the presence of adenosine bound in some form other than 5′-adenylic acid. Since 5′-adenylic acid has the same mobility that DPN has under the conditions employed for electrophoresis, it would not, if present, show up as a separate spot on the ionogram.

Ion Exchange Chromatography of DPN Products—1 gm. of DPN was treated with 5 ml. of TFAA for 14 hours at room temperature, after which the amber solution was evaporated to a viscous gum under reduced pressure. The gum was taken up in 50 ml. of ethyl ether, and the resulting cream-colored precipitate was centrifuged and washed twice with 40 ml. of ether. Considerable fine precipitate settled out of the clear ether supernatant solutions upon standing for 1 hour at room temperature. This was centrifuged and combined with the main precipitate. To the air-dried precipitate 25 ml. of cold absolute ethanol saturated with NH_3 were added. After standing on ice for 20 minutes the heavy cream-colored precipitate was centrifuged, washed with 25 ml. of absolute alcohol, and dissolved in 10 ml. of H_2O. Testing with cyanide showed 90 per cent recovery of the nicotinamide-ribose originally present. Of this amount 11 per cent was

unchanged DPN, as measured by yeast alcohol dehydrogenase. The amount of nicotinamide-ribose split by DPNase in a preliminary trial was 2.5 times as great as the amount of DPN present (as measured by yeast alcohol dehydrogenase).

The solution obtained (pH 9, 17 ml.) was put on a column of Dowex 1 resin, 200 to 400 mesh, in the formate form, 2 cm. in diameter and 4.3 cm. high. The effluent was pale yellow and smelled of ethanol and ammonia. The column was washed with distilled water until the optical density at 260 mμ of a 0.1 ml. aliquot in 3.0 ml. of water fell below 0.1.

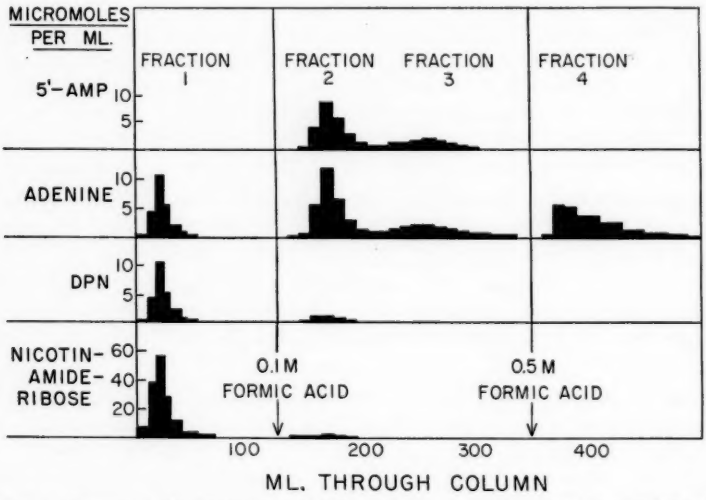


FIG. 1. Dowex 1 chromatogram of the products from the reaction of DPN with TFAA. See the text for identification of the fractions.

The column was then eluted with 0.1 M formic acid, and the progress of the elution was followed by checking the optical density at 260 mμ of 0.1 ml. aliquots of the eluates in 3.0 ml. of H_2O. When this absorption became negligible, the eluent was changed to 0.5 M formic acid. The effluent, washes, and eluates were tested for concentration of nicotinamide-ribose by the cyanide method, for DPN with alcohol dehydrogenase, and for 5′-adenylic acid with the specific 5′-adenylic acid deaminase. Adenine concentration was calculated from optical densities at 260 mμ, correction being made for any absorption at this wave-length due to nicotinamide compounds.

The results of these tests are presented in Fig. 1. Most of the nicotinamide-ribose compounds were not adsorbed on the resin, but came through

in the effluent and washes (Fraction 1). When absorption at 260 mμ due to nicotinamide compounds in this fraction was taken into account, all the adenine in Fraction 1 could be accounted for as DPN. Fraction 2 consisted of 5′-adenylic acid, together with slight amounts of DPN. Fraction 3 was 5′-adenylic acid. The reason for the appearance of this secondary adenylic acid peak is not clear; it was not obtained when this experiment was repeated. Fraction 4 contained adenine, but no nicotinamide-ribose compounds or 5′-adenylic acid. After acidification with 4 N HNO_3, a precipitate was obtained from this fraction with 4 volumes of cold acetone. The precipitate was washed with acetone and ether and then dried over P_2O_5. The yield was 85 mg.

TABLE III

Analysis of $(AR)_2POP$ from Reaction of DPN with TFAA

The reaction mixture contained 0.5 ml. of Fraction 4 from the Dowex 1 column, 0.5 ml. of 1.0 M $NaHCO_3$, 0.05 ml. of 0.33 M $MgCl_2$, and 0.2 ml. of purified snake venom pyrophosphatase or 0.2 ml. of H_2O. After incubation at 37° for 75 minutes, aliquots were analyzed for adenine by measuring optical density at 260 mμ, for 5′-adenylic acid with 5′-adenylic acid deaminase, and for monoester phosphate with prostatic phosphatase. All values are in micromoles.

Compound	Without pyrophosphatase	With pyrophosphatase
Adenine	3.03	3.03
5′-AMP (by deaminase)	0.0	2.92
Monoester phosphate	0.10	3.06

Identification of Fraction 4 As $(AR)_2POP$—The manner in which Fraction 4 was eluted from the column suggested that it contained the same material as the fastest moving component in the electrophoresis pattern of the DPN-TFAA reaction products. The possibility that this material might be $(AR)_2POP$ was tested by analyzing for 5′-adenylic acid after treatment with the snake venom pyrophosphatase. The free adenylic acid liberated was assayed with the specific deaminase of muscle, and, as can be seen from Table III, all the adenine present in the preparation can be accounted for as 5′-adenylic acid after the action of the venom enzyme. The presence of a pyrophosphate linkage is further indicated by the release of inorganic phosphate by the combined action of the pyrophosphatase and the prostatic monoesterase (Table III). These observations allow identification of the material as $(AR)_2POP$.

For comparison $(AR)_2POP$ was prepared from 5′-adenylic acid by the method of Christie *et al.* (15). The procedure involves treating 5′-adenylic acid with TFAA for 3 days at room temperature and separation of the

products by chromatography on Dowex 1 resin. Unchanged 5′-adenylic acid was removed with 0.1 M formic acid, and the $(AR)_2POP$ was eluted with 0.5 M formic acid. The 0.5 M formic acid eluate was acidified with 4 N HNO_3, and the $(AR)_2POP$ was precipitated with 4 volumes of cold acetone, washed with acetone and ether, and dried over P_2O_5. The yield from 500 mg. of 5′-adenylic acid was 72 mg., or 15 per cent, compared with the 17 per cent yield reported by Christie *et al.* (15). Like the corresponding material obtained from DPN, the $(AR)_2POP$ could be deaminated by the intestinal phosphatase preparation, but not by 5′-adenylic acid deaminase.

Isolation and Identification of Nicotinamide Products—To facilitate the analysis of nicotinamide-ribose compounds other than DPN present in Fraction 1, the DPN in this fraction was destroyed. This was accomplished by reducing with yeast alcohol dehydrogenase and then treating the reduced DPN with acid. To do this the pH of Fraction 1 (total volume, 115 ml.) was brought to 10 by the addition of 0.4 ml. of 5 N KOH, and 1.0 ml. of absolute ethanol was added together with 1.0 ml. of a 1:40 dilution of crystalline yeast alcohol dehydrogenase. Reduction, as followed by observing the increase in optical density at 340 mμ, was complete after approximately 8 minutes at room temperature. Then the pH was brought to 3.5 by the addition of 0.9 ml. of 4 N HNO_3, and the solution was incubated for 60 minutes at 37°. At the end of this time destruction of the reduced DPN was complete, as indicated by disappearance of absorption at 340 mμ. The solution was brought to pH 8 with 0.7 ml. of 5 N KOH and then put through a column of Dowex 1 resin in the formate form, 4.5 cm. by 2 cm. in diameter. The column was washed with water until the optical density at 260 mμ of 0.1 ml. in 3 ml. of H_2O fell below 0.1. The effluent and washes were combined, the pH was brought to 5 with 0.5 ml. of 4 N HNO_3, and the solution was concentrated by distillation under reduced pressure at 30° to a volume of 10.7 ml. Some losses were entailed in this procedure, since the yield of nicotinamide-ribose compounds was 635 μmoles. This step did serve, however, to remove completely the DPN.

Paper electrophoresis of the concentrated effluent in 0.05 M ammonium acetate buffer, pH 3.5, with a potential gradient of 20 volts per cm., gave a single spot which moved 2 cm. in the direction of the anode. After chromatography in the ethanol-acetic acid system of Zatman *et al.* (16) two ultraviolet-quenching spots could be detected, both of which fluoresced under ultraviolet light when treated with 1.0 M KCN. The main spot (Component 1) had an R_F of 0.45. The other spot (Component 2) had an R_F of 0.60 and moved at the same rate as did a known sample of nicotinamide mononucleotide.

The two compounds were separated by chromatography in the same system on a larger scale. Bands were identified under ultraviolet light, cut

out, and eluted with 0.01 N HCl for 20 hours at 8°. This elution was successful in removing about 25 per cent of the material applied. The analysis of both eluates is shown in Table IV. In each case the ratio of nicotinamide to ribose to phosphate is approximately 1. This allowed identification of the faster moving component as nicotinamide mononucleotide and strengthened the suspicion that the other compound might be the pyrophosphate condensation product of 2 molecules of NMN.

Like NMN, the faster-moving Component 2 is not split by *Neurospora* DPNase. The slower moving Component 1 is hydrolyzed to the extent of 50 per cent, but at a slower rate than DPN. The action of DPNase on these compounds is compared in Fig. 2. Treatment of the slower moving component with purified snake venom pyrophosphatase causes it to become refractory to hydrolysis by DPNase.

TABLE IV

Analysis of Chief Nicotinamide Products from Reaction of DPN with TFAA

All values are in micromoles. The ribose values are based on DPN as a standard.

Compound	Component 1	Component 2
Nicotinamide-ribose	1.05	0.95
Ribose	0.92	0.83
Total phosphate	1.00	0.89

Chromatography after treatment of this component with the venom pyrophosphatase yielded a single spot which moved at the same rate that both nicotinamide mononucleotide and the original fast moving component do (Fig. 3).

On the basis of these results the slower moving component has been identified as dinicotinamide-riboside-5′-pyrophosphate ($(NR)_2POP$). As estimated from the results of treatment with DPNase, this compound accounts for about two-thirds of the nicotinamide-ribose linkages in the DPN-TFAA reaction products, while most of the remainder consists of nicotinamide mononucleotide.

DPNase removes only one nicotinamide group of $(NR)_2POP$.[3] The product of the action of DPNase on $(NR)_2POP$ was isolated by chromatography in the ethanol-acetic acid system and analyzed for nicotinamide, ribose, and phosphate. The results of this analysis (Table V) agree with the predicted formulation of this product as nicotinamide-ribose-5′-pyrophosphate-5-ribose.

The balance of the products of the reaction of DPN with TFAA is shown

[3] The DPNases from pig brain and beef spleen as well as the *Neurospora* enzyme have been found to split $(NR)_2POP$.

in Table VI. Yields obtained when this experiment was repeated were somewhat lower than those presented here. From these data it appears that the fate of each half of the DPN molecule in this reaction is the same. About one-third remains as the free mononucleotide, while most of the remainder goes to form the pyrophosphate condensation product. There is also the possibility that part of the DPN present may be formed by recombination of the two mononucleotide split-products.

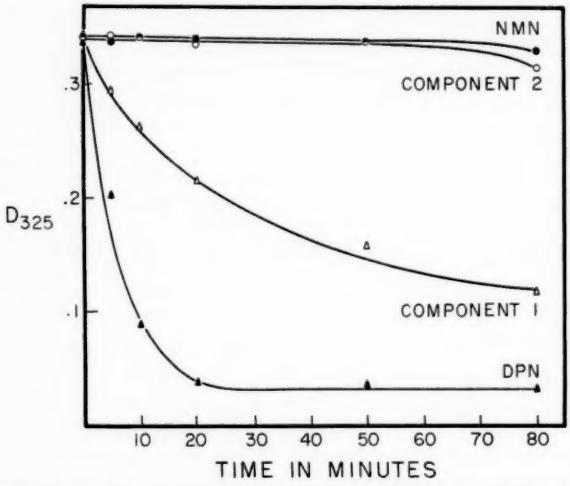


FIG. 2. Time curve for comparing the action of *Neurospora* DPNase on NMN, DPN, and the nicotinamide-ribose products of the DPN-TFAA reaction. The reaction mixture contained 0.4 μmole of nicotinamide-ribose, 7 mg. of sodium acetate, 0.1 ml. of enzyme, and water to a total volume of 0.6 ml. This was incubated at 37°, and, at various times, aliquots of 0.08 ml. were withdrawn into 1.0 ml. of 1.0 M KCN and the amount of nicotinamide-ribose present was determined by measuring the optical density at 325 mμ in a 1 ml. cuvette.

Exchange Reaction between DPN and Inosinic Acid—25 mg. of DPN and 100 mg. of 5′-inosinic acid (Ba salt) were placed in a 12 ml. glass centrifuge tube, and 0.5 ml. of trifluoroacetic acid anhydride was added; the clear solution was allowed to stand 18 hours at room temperature. Most of the TFAA was removed by evaporation under reduced pressure, and the residual gum was triturated in ether to a dry powder. This powder was treated with cold absolute ethanol saturated with ammonia, then washed with absolute ethanol. The precipitate was suspended in 1 ml. of H_2O and brought into solution by adding HCl until the pH reached 4. Barium ions were then precipitated with 0.04 ml. of 0.5 M K_2SO_4, and the super-

natant fluid was brought to pH 8.0. Approximately 25 μmoles of nicotinamide-ribose were obtained in this preparation as determined by the cyanide procedure. Of this, about 6 μmoles were deamino-DPN as indi-

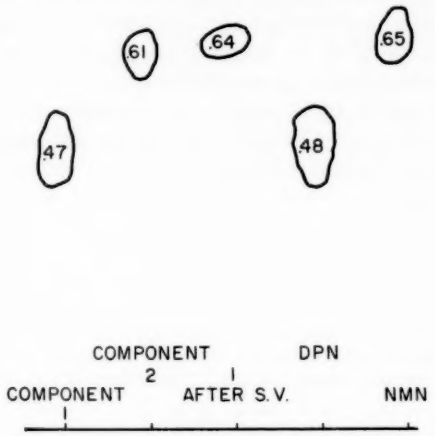


FIG. 3. Identification of nicotinamide products from the reaction of DPN with TFAA. An aliquot of the slower moving nicotinamide component containing 1 μmole of nicotinamide-ribose was incubated at 37° for 1 hour with 0.1 ml. of 0.1 M $MgCl_2$, 0.07 ml. of 1.0 M tris(hydroxymethyl)aminomethane buffer, pH 8.1, and 6.8 mg. of lyophilized snake venom (S. V.) pyrophosphatase in a total volume of 0.7 ml. After HCl was added to lower the pH to 4.5, the reaction mixture was boiled for 1 minute and an aliquot was chromatographed on No. 3 Whatman paper. The solvent consisted of equal parts of ethanol and 0.1 M acetic acid. The numbers in the spots represent the R_F values.

TABLE V

Analysis of $(NR)_2$POP after Treatment with DPNase

The incubation mixture contained 3.8 μmoles of $(NR)_2$POP and 0.04 ml. of *Neurospora* DPNase in 10 ml. of 0.1 M acetate buffer, pH 5.0. After 30 minutes at 37°, hydrolysis of one-half the nicotinamide-ribose linkages, as determined by testing with cyanide, was complete. The mixture was then boiled for 2 minutes and passed through a column of Dowex 1 resin in the formate form. The effluent and washes were combined, concentrated, and analyzed for total phosphate and for ribose as described in the text.

		Ratio to nicotinamide-riboside
	μmoles	
Nicotinamide-ribose	1.92	1.0
Phosphate	3.50	1.8
Ribose	3.40	1.8

cated by the rate of reaction with yeast alcohol dehydrogenase.[4] These results show that an exchange between inosinic acid and the adenylic acid moiety of DPN occurred in the presence of TFAA.

Synthesis of Pyrophosphate Linkage of DPN—50 mg. of dry NMN and 50 mg. of 5′-adenylic acid (AMP) or $(AR)_2POP$ were treated with 0.5 ml. of TFAA. After 16 hours at room temperature both reaction mixtures were in the form of an amber gel which melted readily when warmed. The TFAA was removed by evaporation. Upon addition of ether, satisfactory precipitates were formed only after standing several hours at room temperature. Absolute alcohol saturated with NH_3 was added to the air-dried ether precipitates. After 10 minutes in ice the precipitates were

TABLE VI

Balance of Reaction Products of DPN with TFAA

The amount of adenine is calculated from optical densities at 260 mμ, corrected for absorption at this wave-length, which can be ascribed to nicotinamide-ribose compounds. Values for NMN and $(NR)_2POP$ are estimated from results obtained with DPNase and paper chromatography. All values are in micromoles.

	Adenine		Nicotinamide-riboside	
		per cent total		*per cent total*
Total	890		1031	
DPN	172	19.2	172	16.7
NMN			275	26.6
$(NR)_2POP$			550	53.3
5′-AMP	344	39.0		
$(AR)_2POP$	330	37.0		

centrifuged and washed with cold absolute ethanol. The precipitates were dissolved in about 4 ml. of H_2O to give a clear amber solution, pH 8, and assayed for DPN by yeast alcohol dehydrogenase.

The yield of DPN from AMP and NMN was 18 per cent. Somewhat less DPN was obtained with the $(AR)_2POP$ and NMN, the yield being about 10 per cent. These results indicate that there is a greater tendency for the formation of $(NR)_2POP$ than for the synthesis of DPN.

DISCUSSION

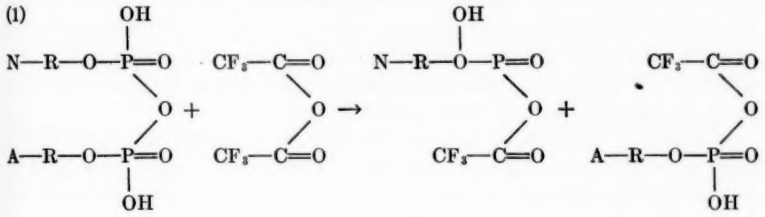
Trifluoroacetic acid anhydride has been employed as a catalyst for the acylation of hydroxyl groups and for the condensation of acids to form anhydrides (18). The cyclization of 2′- and 3′-mononucleotides (2) and the condensation of 5′-mononucleotides to dinucleoside pyrophosphates (15)

[4] Deamino-DPN reacts at approximately one-seventh the rate of DPN with yeast alcohol dehydrogenase (17).

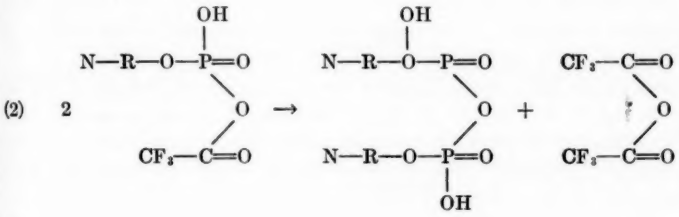
have also been carried out by the use of TFAA. The intermediates in these reactions appear to be mixed anhydrides of the reacting acid with trifluoroacetic acid, and the basic mechanism involved is apparently one of anhydride exchange.

It now appears that the condensation of phosphate monoesters to pyrophosphates under the influence of TFAA is a reversible reaction. The observed reactions of DPN and TFAA may be explained by a mechanism involving selective cleavage of the pyrophosphate linkage to yield 5′-adenylic acid, nicotinamide mononucleotide, and their trifluoroacetyl anhydrides. These may then recombine to form $(AR)_2POP$, $(NR)_2POP$, and some of the original mixed pyrophosphate, DPN.

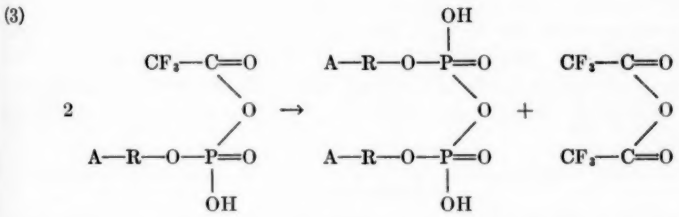
(1)



(2)



(3)



These reactions are summarized in the accompanying scheme. Alcoholysis during the isolation of the products would release 5′-adenylic acid and NMN from their trifluoroacetyl anhydrides. If the formation of $(AR)_2POP$ were to require 2 molecules of trifluoroacetyl-5′-adenylic acid, this might help explain the low yields obtained when 5′-adenylic acid is treated with TFAA, since in this case only 1 molecule of mixed anhydride could be formed per molecule of TFAA. It is possible that the somewhat

larger yields of $(AR)_2POP$ obtained when TFAA reacts with DPN than with 5′-AMP may be due to a greater formation of the trifluoroacetyl-5-adenylic acid. This would indicate that the anhydrides such as DPN have a greater tendency to react with TFAA than do the free mononucleotides. Starting with an anhydride such as DPN would also avoid the release of free trifluoroacetic acid, which could also interfere with the formation of $(AR)_2POP$.

These reactions open the way to the synthesis of a new series of DPN analogues. For example, the uracil analogue of DPN (U—R—P—P—R—N) might be formed by the reaction of 5′-uridylic acid with either DPN or NMN in the presence of TFAA. Similarly the adenylic acid ribose of DPN could be replaced by deoxyribose. Analogues of $(NR)_2POP$ in which one nicotinamide is replaced by some other pyridine derivative might possibly be formed by the enzymatic exchange reaction of Zatman *et al.* (19), since the DPNases from beef spleen and pig brain split $(NR)_2POP$ in the same manner as does the *Neurospora* DPNase.

It is also of interest to mention some preliminary experiments on the reaction of riboflavin-5-phosphate and 5′-AMP in the presence of TFAA. A product was obtained which is active in the D-amino acid oxidase reaction. This enzyme is inactive with riboflavin-5-phosphate and is reported to be specific for flavin adenine dinucleotide. A detailed investigation of the reactions of flavin nucleotides with TFAA is now in progress.

The reaction of TPN with TFAA seems to be similar to that of DPN. Because of the known ability of TFAA to isomerize and cyclize 2′- and 3′-mononucleotides, at least six possible diadenosine pyrophosphate compounds could be obtained. The evidence available indicates that the nicotinamide products formed resemble those obtained from DPN.

The splitting of $(NR)_2POP$ by *Neurospora* DPNase is noteworthy, since it is cleaved at about one-third of the rate of DPN, but much faster than deamino-DPN (20). Mr. N. Bachur in this laboratory has found that the compound can replace DPN as a growth factor for *Hemophilus parainfluenzae*. Details of the biological activity of $(NR)_2POP$ will be described elsewhere.

SUMMARY

1. Trifluoroacetic acid anhydride (TFAA) selectively cleaves the pyrophosphate linkage of DPN and TPN, but leaves the nicotinamide-ribose linkage largely intact.

2. The chief products of the reaction of TFAA with DPN have been isolated and identified as 5′-adenylic acid (5′-AMP), nicotinamide mononucleotide (NMN), diadenosine-5′-pyrophosphate ($(AR)_2POP$), and dinicotinamide-ribose-5′-pyrophosphate ($(NR)_2POP$). The yields of ade-

nine in 5′-AMP and $(AR)_2POP$ are approximately equal, while about twice as much $(NR)_2POP$ is formed as is NMN.

3. The pyrophosphate linkages of both $(AR)_2POP$ and $(NR)_2POP$ are split by snake venom pyrophosphatase to give the corresponding mononucleotides.

4. $(NR)_2POP$ is hydrolyzed by *Neurospora* DPNase about one-third as rapidly as DPN. The DPNase removes only one nicotinamide group from the $(NR)_2POP$ molecule.

5. In the presence of trifluoroacetic acid anhydride, NMN has been found to react with 5′-AMP or $(AR)_2POP$ to yield DPN.

6. The reaction mechanisms involved are discussed and their application to the synthesis of new analogues of DPN is considered.

BIBLIOGRAPHY

1. Shuster, L., and Kaplan, N. O., *J. Biol. Chem.*, **215**, 183 (1955). Wang, T. P., Shuster, L., and Kaplan, N. O., *J. Biol. Chem.*, **206**, 299 (1954).
2. Brown, D. M., Magrath, D. I., and Todd, A. R., *J. Chem. Soc.*, 2708 (1952).
3. Markham, R., and Smith, J. D., *Biochem. J.*, **52**, 558 (1952).
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
5. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., *J. Biol. Chem.*, **191**, 447 (1951).
6. Taylor, J. F., Velick, S. F., Cori, G. T., Cori, C. F., and Slein, M. W., *J. Biol. Chem.*, **173**, 619 (1948).
7. Racker, E., *J. Biol. Chem.*, **184**, 313 (1950).
8. Grafflin, A. L., and Ochoa, S., *Biochim. et biophys. acta*, **4**, 205 (1950).
9. Nikiforuk, G., and Colowick, S. P., in Colowick, S. P., and Kaplan, N. O., Methods in enzymology, New York, in press.
10. Kaplan, N. O., Colowick, S. P., and Nason, A., *J. Biol. Chem.*, **191**, 473 (1951).
11. Hurst, R. O., and Butler, G. C., *J. Biol. Chem.*, **193**, 91 (1951).
12. Shuster, L., and Kaplan, N. O., in Colowick, S. P., and Kaplan, N. O., Methods in enzymology, New York, in press.
13. Brown, D. M., and Todd, A. R., *J. Chem. Soc.*, 44 (1952).
14. Burton, R. M., and San Pietro, A., *Arch. Biochem. and Biophys.*, **48**, 184 (1954).
15. Christie, S. M. H., Elmore, D. T., Kenner, G. W., Todd, A. R., and Weymouth, F. J., *J. Chem. Soc.*, 2947 (1953).
16. Zatman, L. J., Kaplan, N. O., and Colowick, S. P., *J. Biol. Chem.*, **200**, 197 (1953).
17. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, **194**, 593 (1952).
18. Bourne, E. J., Stacey, M., Tatlow, J. C., and Tedder, J. M., *J. Chem. Soc.*, 2976 (1949).
19. Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M., *J. Biol. Chem.*, **209**, 467 (1954).
20. Kaplan, N. O., Colowick, S. P., and Ciotti, M. M., *J. Biol. Chem.*, **194**, 579 (1952).

THE STRUCTURE OF CEREBROSIDE SULFURIC ESTER OF BEEF BRAIN*

By S. J. THANNHAUSER, JOSEF FELLIG,† and GERHARD SCHMIDT

(From the Research Laboratory of the Boston Dispensary, New England Medical Center, Tufts College Medical School, Boston, Massachusetts)

(Received for publication, December 15, 1954)

A cerebroside sulfuric ester (CSE) was first isolated in 1933 by Blix (1) from beef brain, and its constituents were described as cerebronic acid, sphingosine, and galactose. The position of the sulfate group was not determined, but Blix suggested that it might be esterified with the galactose. In 1953 a method for the isolation of pure cerebroside sulfuric ester from beef brain was described by Thannhauser and Boncoddo (2). The purpose of the present paper is to determine the position of the sulfuric ester in the cerebroside molecule. To that effect CSE was completely methylated, and the methylgalactose obtained after splitting was identified on the paper chromatogram by comparison to known methylgalactoses.

Preparation of CSE

25 pounds of acetone-dried beef brain powder were stirred with 10 liters of 95 per cent ethanol at 37° for 3 days. The suspension was filtered over a Büchner funnel, and the precipitate was washed twice with 95 per cent ethanol at room temperature and three times with ether. The dry solids were extracted with ether in a Soxhlet apparatus for 3 days. The thimbles containing the residual material were suspended by means of a wire loop in the wide neck of round bottomed flasks containing 90 per cent acetone and extracted by refluxing for 24 hours. The acetone extract which contains cerebroside and CSE was taken to dryness under reduced pressure. The residue (115 gm.) was dissolved in 580 ml. of pyridine at 60° and placed in the refrigerator overnight. The white, crystalline precipitate was filtered off, and the precipitation was repeated twice with one-half and one-fourth of the original volume of pyridine, respectively. This procedure eliminated the bulk of the cerebrosides. The final precipitate was washed with ether three to four times and dried. The remaining white material

* This study was aided by grants from the Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Neurological Diseases and Blindness, the Godfrey M. Hyams Trust Fund, and the Charlton Fund.

† Present address, Division of Biochemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts.

XUM

(30.5 gm.) was dissolved in 600 ml. of warm pyridine and placed on an alumina column (Alcoa F-20) (200 × 25 mm.). The effluent pyridine was passed through a second column of the same dimensions. The CSE is adsorbed on the alumina, while most of the cerebroside remains in the pyridine. These adsorptions should be carried out at approximately 35°. The alumina of both columns was extracted in a Soxhlet apparatus with chloroform-methanol (2:1) for 1 day to remove traces of adsorbed cerebroside. The alumina was then placed in a paper thimble and extracted directly with boiling 90 per cent acetone for 1 week as described above. The acetone was taken to dryness under reduced pressure and the residue (3.6 gm.) recrystallized twice from 5 volumes of hot chloroform-methanol (2:1). 2.15 gm. of a white crystalline product were obtained.

$C_{48}H_{92}O_{12}NS$.	Calculated.	C 62.6, H 10.1, S 3.5
	Found.	" 62.22, " 10.86, " 3.25 (corrected for ash)

Methylation of CSE

4.2 gm. of CSE were dissolved in 180 ml. of chloroform-methanol (3:1). 40 ml. of methyl iodide and 12 gm. of freshly prepared silver oxide were added, and the mixture was shaken for 6 hours. The silver oxide was filtered off and extracted with a small amount of boiling chloroform, which was added to the filtrate. The solution was taken to dryness under reduced pressure and the methylation repeated eight times. The addition of methanol was necessary for only the first and second methylations, since the partly methylated CSE is soluble in chloroform. Yield, 2.8 gm. Analysis (after recrystallization from ethanol):

$C_{53}H_{102}O_{12}NS$.	Calculated.	C 60.9, H 10.4, OCH_3 15.8.
	Found.	" 61.2, " 10.09, " 14.72
		15.33 (different batch)

Methanolysis of Methylated CSE and Separation of Components

2.8 gm. of methylated CSE were heated for 6 hours at 100° in a pressure bottle with 80 ml. of anhydrous methanol containing 8 per cent HCl. The solution was placed in the refrigerator overnight. The precipitated methyl esters of the fatty acids were filtered off, and the methanol was extracted three times with petroleum ether to remove traces of fatty acid esters. The filtrate was taken to dryness under reduced pressure with methanol five times to eliminate the hydrochloric acid. The residue was dissolved in 40 ml. of water and brought to pH 9.0 with 0.1 N NaOH. The sphingosine precipitated on standing in the refrigerator; it was removed by centrifugation in the cold. The supernatant fluid, which did not give a reaction with ninhydrin, was adjusted to pH 6.5 with 0.1 N HCl and the solution taken to

dryness under reduced pressure. The residue was dried *in vacuo* and extracted twice with 10 ml. of ether and three times with 10 ml. of chloroform. The combined solutions were taken to dryness. The remaining methylated methylgalactoside was dissolved in 10 ml. of 5 per cent HCl and heated for 6 hours at 100° in a pressure bottle to prepare the free methylated sugar. The methylated galactose which was obtained as a syrup was examined by paper chromatography (Fig. 1). It consisted almost entirely of 2,3,4-trimethylgalactose, a small amount of 2,4-dimethylgalactose being present because of the difficulties inherent to complete methylation. No 2,3,4,6-tetramethylgalactose was found. This finding indicated that the sulfuric acid in CSE is esterified to the primary hydroxyl of carbon atom 6 in the galactose moiety of the molecule.

Preparation of Reference Sugars

2,3,4,6,-Tetramethylgalactose—This was obtained by the methylation of beef brain cerebrosides (3). Methylation, hydrolysis, and isolation were carried out as described above for CSE. Because of incomplete methylation a trace of 2,3,4-trimethylgalactose was present in the material.

2,3,4-Trimethylgalactose—6-Trityl-β-methylgalactoside was prepared according to Müller (4) from β-acetobromogalactose. It was dissolved in chloroform and methylated with methyl iodide and silver oxide at room temperature. The methylation was completed by refluxing the partially methylated material in 5 times its weight of methyl iodide in a 3-necked flask, equipped with a condenser and a mercury seal stirrer, on a water bath for 3 hours. Freshly prepared silver oxide (4 times the weight of the material) was added in small portions during the course of the methylation. This methylation was repeated three times. 8 gm. of the methylated material were dissolved in 50 ml. of 90 per cent ethanol, to which 2 ml. of concentrated hydrochloric acid were added, and refluxed for 2 hours. After cooling, the solution was poured into 250 ml. of water and the precipitated triphenylcarbinol filtered off. The filtrate was concentrated to 20 ml. under reduced pressure, the pH adjusted to 5.0 by silver carbonate, and the precipitate filtered. The filtrate was acidified by dilute acetic acid, and the silver was removed with hydrogen sulfide. The silver sulfide was filtered and the supernatant fluid taken to dryness under reduced pressure. The residue was taken up in ether and filtered. 3.5 gm. of trimethyl-β-methylgalactoside were obtained after removal of the ether. The methylgalactoside was distilled in high vacuum (b.p. 95–96° at 1 mm. of Hg). The free methylated sugar was obtained by hydrolysis in 5 per cent HCl at 100° for 6 hours in a pressure bottle. The hydrochloric acid was removed as indicated above and the residue taken up in a chloroform-ether (1:1) mixture. After removal of the solvents a syrup was obtained which solid-

ified after prolonged drying in high vacuum. The methoxyl content of the very hygroscopic solid was determined.

$C_9H_{18}O_6$. Calculated, OCH_3 41.8; found, OCH_3 40.87

The anilide was prepared from the dried material, m.p. 163° (uncorrected) (reported melting point for 2,3,4-trimethylgalactose anilide, 165–167° (5)).

Fatty Acid and Sphingosine Fractions

The fatty acid esters which were obtained after methanolysis of methylated CSE were dried and distilled in high vacuum in a microdistillation apparatus. Three fractions were obtained and the free fatty acids prepared from them by saponification. After recrystallization from acetone, the melting points of the free acids were 75°, 85°, and 88°, respectively. The methoxyl content of the highest and lowest melting fatty acid was 6.92 and 6.73 per cent, respectively (calculated for methoxyl lignoceric acid (methylcerebronic acid), 7.7 per cent). The slightly low methoxyl content may be due to incomplete methylation of the cerebronic acid or to the presence of small amounts of lignoceric acid.

The sphingosine fraction was dissolved in absolute ethanol and the solution acidified by the dropwise addition of methanolic sulfuric acid and placed in the refrigerator. The *O*-methyl ether of sphingosine sulfate crystallized out and was centrifuged. The analysis showed it to be the acid salt of *O*-methylsphingosine.

$C_{19}H_{39}O_2N \cdot \frac{1}{2}H_2SO_4$.	Calculated.	N 4.1,	OCH_3 8.5
$C_{19}H_{39}O_2N \cdot H_2SO_4$.	"	" 3.4,	" 7.5
	Found.	" 3.74,	" 7.43

DISCUSSION

The problem of the position of the sulfate group in CSE has previously been examined by Nakayama (6). He attempted unsuccessfully to prepare the triphenyl methyl ester of CSE. From the failure of his material to react with trityl chloride, he concluded that the primary hydroxyl of the galactose was esterified by the sulfuric acid. However, when we tried to tritylate beef brain cerebrosides under the same and under more vigorous conditions, we could not obtain a tritylated reaction product. For this reason the negative evidence of Nakayama does not permit any conclusions as to the structure of CSE.

The methylation of our CSE was carried out with methyl iodide and silver oxide at room temperature under conditions previously described for free cerebrosides (3). It was found, however, from the methoxyl determinations as well as from the paper chromatograms that the methylation

did not proceed to completion, even when it was repeated eight times. We therefore attempted to complete the methylations at room temperature by a subsequent methylation at the temperature of refluxing methyl iodide. It was found, however, that under these conditions the sulfate ester of CSE was partially split and the method was therefore unsuitable for our purposes. When the methylated galactose from CSE was examined on the paper chromatogram (Fig. 1), it was found that the main product is 2,3,4-trimethylgalactose. A small amount of 2,4-dimethylgalactose is present because of the incomplete methylation mentioned above.

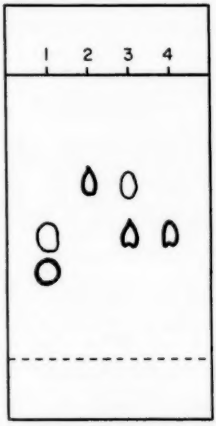


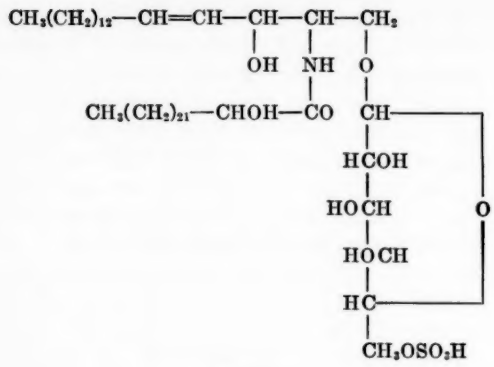
FIG. 1. Descending chromatogram (20 hours). Solvent, *n*-butanol-ethanol-water, 5:1:4 (organic phase) (8). Reagent, aniline phthalate. (*1*) 2,3,4,6-Tetramethylgalactose from methylated cerebroside; (*2*) 2,4-dimethylgalactose, synthetic; (*3*) methylated galactose from cerebroside sulfuric ester; (*4*) 2,3,4-trimethylgalactose, synthetic.

Of much greater importance, however, is the complete absence of 2,3,-4,6-tetramethylgalactose in the methylated galactose from CSE, in contrast to the material obtained under the same conditions from beef brain cerebrosides. This shows unequivocally that the sulfuric acid in CSE must be esterified to the primary hydroxyl on carbon atom 6 of the galactose.

The fatty acid fraction seems to consist essentially of cerebronic acid, in confirmation of the findings of Blix (1). On the basis of Carter and Greenwood's (7) structure for cerebrosides we can assign the structure to cerebroside sulfuric ester (see the diagram).

It should be mentioned that our CSE preparation had no anticoagulant activity.[1]

We are indebted to Dr. Mario Stefanini for testing this material.



SUMMARY

A method for the preparation of cerebroside sulfuric ester is described. The position of the sulfuric acid group in cerebroside sulfuric ester was shown to be on the primary hydroxyl of carbon atom 6 of the galactose moiety of the molecule.

We gratefully acknowledge the assistance of Mr. N. F. Boncoddo, Mr. R. Bonofiglio, Mr. M. J. Bessman, and Mrs. M. D. Hickey.

BIBLIOGRAPHY

1. Blix, A., *Z. physiol. Chem.*, **219**, 82 (1933).
2. Thannhauser, S. J., and Boncoddo, N., *Federation Proc.*, **12**, 280 (1953).
3. Pryde, J., and Humphreys, R. W., *Biochem. J.*, **18**, 661 (1924).
4. Müller, A., *Ber. chem. Ges.*, **64**, 1820 (1931).
5. Bell, D. J., *Advances in Carbohydrate Chem.*, **6**, 23 (1951).
6. Nakayama, T., *J. Biochem.*, Japan, **38**, 157 (1951).
7. Carter, H. E., and Greenwood, F. L., *J. Biol. Chem.*, **199**, 283 (1952).
8. Jones, J. K. N., *Discussions Faraday Soc.*, **7**, 271 (1949).

SYNTHETIC α-AMINO-β-HYDROXYCAPROIC ACIDS

By H. W. BUSTON and JILLIAN BISHOP

(From the Biochemical Laboratories, Department of Botany, Imperial College of Science, London, England)

(Received for publication, December 20, 1954)

In a previous communication (1) a synthesis of the two diastereoisomers of α-amino-β-hydroxy-*n*-valeric acid was reported. By similar methods certain hydroxyaminocaproic acids (hydroxyleucines) have now been prepared.

EXPERIMENTAL

α-Amino-β-hydroxy-n-caproic Acids (Hydroxynorleucines)—From 55 gm. of β-*n*-propylacrylic acid (m.p. 33°) by treatment in cold aqueous solution with bromine in a current of air, 37 gm. of α-bromo-β-hydroxy-*n*-caproic acid were obtained (m.p. 87–88°; Br 37.80 per cent, calculated for $C_6H_{11}O_3Br$, 37.89 per cent). 36 gm. of the bromo acid, aminated in cold aqueous solution with excess of ammonium hydroxide for 10 days, gave 15 gm. of amino acid, which crystallized from dilute ethanol in elongated tetragonal prisms. A paper chromatogram in water-butanol-diethylamine (Hardy and Holland (2)) revealed the presence of only one amino acid. Analysis gave C 49.16, H 9.00, N 9.37 per cent; required for $C_6H_{13}O_3N$, C 49.00, H 8.84, N 9.52. Treatment with periodic acid liberated *n*-butyraldehyde, confirming the product as an α-amino-β-hydroxy acid.

The diastereoisomer (Type II) of this amino acid was prepared by the method of Attenburrow, Elliott, and Penny (3); it crystallized from dilute ethanol in large asymmetric hexagonal plates. Found, C 49.14, H 9.09, N 9.52 per cent. Further data concerning these two amino acids are reported in Table I.

α-Amino-β-hydroxyisocaproic Acids (Hydroxyleucine)—β-Isopropylacrylic acid was prepared from isobutyraldehyde and purified by the partial esterification method of Linstead (4). This purification was essential, as the isopropylacrylic acid (a liquid) was always contaminated with a considerable amount of the $\Delta^{\beta,\gamma}$ isomer.

From 50 gm. of isopropylacrylic acid, 30 gm. of the bromohydroxy acid were obtained (m.p. 93°; Br 37.6 per cent), and amination of 20 gm. of this acid gave 5 gm. of amino acid which crystallized in short, thick, hexagonal prisms. A paper chromatogram showed a single ninhydrin-sensitive spot, and treatment with periodic acid liberated isobutyraldehyde. Analysis gave C 48.84, H 8.87, N 9.64 per cent. The diastereoisomer (Type

II) was prepared as before and crystallized in thin hexagonal plates. Found, C 48.68, H 8.42, N 9.79 per cent.

Biological Properties—Tests were carried out in order to discover whether any antagonism could be demonstrated between the hydroxyamino acids and their non-hydroxy analogues for the growth of microorganisms. The

TABLE I

Isomeric α-Amino-β-hydroxy-n-caproic Acids

	Hydroxynorleucine		Hydroxyleucine	
	Type I	Type II	Type I	Type II
M.p., °C.	216	209	240	209
" of *N*-benzoyl compound, °C.	186	157	196	184
R_F value (water-butanol-diethylamine)	0.45	0.51	0.41	0.48

TABLE II

Antagonism between DL-*Threonine and Hydroxy-*DL-*norleucine (Type II) for Growth of S. faecalis*

Incubated for 72 hours at 37°.

DL-Threonine	Hydroxy-DL-norleucine	Acid produced equivalent to
γ per 10 ml.	*γ per 10 ml.*	*ml. 0.1 N NaOH*
0	0	1.60
0	80	1.62
0	320	1.57
20	0	3.37
20	40	3.28
20	80	3.15
20	320	2.38
40	0	4.12
40	320	3.63
80	0	4.63
80	160	4.10

only instances of antagonism found were (1) between hydroxyleucine (Type II) and leucine for the growth of *Lactobacillus arabinosus*, and (2) a rather slight antagonism between hydroxynorleucine (Type II) and threonine for the growth of *Streptococcus faecalis*. The present results are parallel with those previously recorded for the hydroxyvalines, but it may be noted that hydroxynorleucine shows considerably less inhibition towards threonine than does hydroxynorvaline (1). In no instance could a hydroxyleucine replace leucine or threonine in nutrient media. Typical re-

sults are recorded in Tables II and III, and Table IV summarizes the results of the biological tests in the present series of experiments.

On both chemical and biological grounds it is concluded that the hydroxynorleucine designated Type II is structurally related to threonine.

TABLE III

Antagonism between L-*Leucine and Hydroxy-*DL-*leucine (Type II) for Growth of L. arabinosus*

Incubated for 72 hours at 30°.

L-Leucine	Hydroxy-DL-leucine	Acid produced equivalent to
γ per 10 ml.	*γ per 10 ml.*	*ml. 0.1 N NaOH*
0	0	1.40
0	40	1.42
0	80	1.30
0	240	1.55
20	0	5.95
20	40	5.20
20	240	4.52
40	0	9.82
40	40	7.05
40	240	5.60
40	480	4.66
80	0	13.91
80	240	11.30

TABLE IV

Antagonism between Amino Acids and Hydroxyamino Acids for Growth of Microorganisms

	Hydroxy-valine	Hydroxy-norvaline		Hydroxy-leucine		Hydroxy-norleucine	
		Type I	Type II	Type I	Type II	Type I	Type II
L-Valine (*L. arabinosus*)	+	−	−	*	−	*	−
L-Leucine " "	−	*	−	−	+	−	−
L-Threonine (*S. faecalis*)	−	−	+	−	−	−	+

+, antagonism demonstrated; −, no antagonism demonstrated.
* Not investigated.

SUMMARY

Two stereoisomeric forms of DL-α-amino-β-hydroxy-*n*-caproic acid and two of DL-α-amino-β-hydroxyisocaproic acid have been prepared, and some of their chemical and biological properties are recorded.

BIBLIOGRAPHY

1. Buston, H. W., Churchman, J., and Bishop, J., *J. Biol. Chem.*, **204**, 665 (1953).
2. Hardy, T. L., and Holland, D. O., *Chem. and Ind.*, 855 (1952).
3. Attenburrow, J., Elliott, D. F., and Penny, G. F., *J. Chem. Soc.*, 310 (1948).
4. Linstead, R. P., *J. Chem. Soc.*, 2498 (1929).

BIOCHEMICAL STRUCTURE OF MITOCHONDRIA

I. INTRAMITOCHONDRIAL COMPONENTS AND OXIDATIVE PHOSPHORYLATION*

By PHILIP SIEKEVITZ† and VAN R. POTTER

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisconsin)

(Received for publication, December 21, 1954)

In the past 10 years, many papers have appeared relating to the problem of the extreme lability of the oxidative phosphorylation system in homogenates and in mitochondria ((2), *cf.* (3)). It has been found that the phosphorylation could not be restored by the addition of any of the known cofactors, and it was therefore impossible to decide whether enzymes or coenzymes or both had been lost (4, 5). Recently (6, 7), we reported experiments in which rat liver mitochondria, briefly incubated at 30°, exhibited marked alterations in their apparent ATPase[1] activity and in their response to DNP and other additions. These alterations are experimentally associated with a loss in the ability to carry out oxidative phosphorylation as observed in this laboratory and elsewhere (*cf.* (3)). Concomitantly, experiments on the regulation of oxidative phosphorylation in the resting mitochondria (8) indicated indirectly that the relative amounts of the adenine nucleotides within the mitochondria could vary independently of the relative amounts in the surrounding medium. In this and the following paper (9), it is shown that the mitochondria contain constant amounts of nucleotides and magnesium which are probably bound, that some of these compounds are active in oxidative phosphorylation, and that mitochondria which have lost all or some of these compounds are no longer capable of coupling phosphorylation to oxidation. The present paper reports data mentioned earlier (1) on the nature of the intramitochondrial

* This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and in part by an institutional research grant (No. 71) from the American Cancer Society. A portion of this work was presented at the meeting of the American Society of Biological Chemists, Chicago, April 8, 1953 (1).

† Present address, Laboratories of The Rockefeller Institute for Medical Research, New York.

[1] Abbreviations are used as follows: AMP, ADP, ATP = adenosine mono-, di-, and triphosphates; DPN and TPN = diphospho- and triphosphopyridine nucleotides; DNP = 2,4-dinitrophenol; PCA and TCA = perchloric and trichloroacetic acids; IP and PP = inorganic orthophosphate and pyrophosphate; IMP and ITP = inosine mono- and triphosphates; FAD = flavin adenine dinucleotide; UDX = derivative of uridine diphosphate; Mt = mitochondria.

nucleotides and on the conditions under which these nucleotides are released to the medium, with subsequent loss in the oxidative phosphorylative ability of the mitochondria. Since this work was carried out, a short note by Lindberg and Ernster (10) has reported restoration of oxidative phosphorylation in mitochondria briefly treated with calcium, but the status of the intramitochondrial nucleotides was not studied in that work.

EXPERIMENTAL

Mitochondria from the livers of fed, 100 to 150 gm. male rats (obtained from the Holtzman Rat Company, Madison) were prepared by the method of Schneider and Hogeboom (11) as modified by Siekevitz and Potter (12). The mitochondria were taken up in cold isotonic (0.25 M) sucrose solution for use. The term equivalent mg. of mitochondria refers to that amount of mitochondria from the given wet weight of liver.

In the experiments determining the release of acid-soluble compounds absorbing maximally at 260 mμ from incubated mitochondria ("E_{260} release"), 500 or 1000 equivalent mg. of mitochondria were incubated in isotonic sucrose solution at 30° in the presence of different components, the total volume being 4 ml. At the end of the incubation, the suspension of mitochondria was spun at 7500 × g for 10 minutes in the International refrigerated centrifuge. Since the resulting supernatant fluid contained soluble protein, 2.0 ml. of 1.5 N PCA were added, the denatured protein was centrifuged, and the resulting supernatant solution was read at 260 mμ in the Beckman DU spectrophotometer to obtain the amount of E_{260} release from the mitochondria.

In many experiments, the amount of acid-soluble material absorbing at 260 mμ that remained in the mitochondria was also determined, and, when this amount was added to the amount released, the total corresponded closely to the amount at zero time (see Figs. 1 and 2). This measurement of mitochondrial E_{260} material was necessary when adenine nucleotides were added to the reaction mixture, since this addition made determination of the E_{260} release less accurate. This acid-soluble extract of the mitochondria was prepared by adding 6.0 ml. of 1.5 N PCA to the pellet of the centrifuged mitochondria after an initial wash of the pellet with isotonic sucrose solution. The mitochondrial suspension was then homogenized and allowed to stand for 1 hour in the cold before centrifuging the protein precipitate. The experiments in which oxidative phosphorylation was measured simultaneously with the E_{260} release are described in Figs. 5 and 6 and Table I.

The magnesium concentration of the mitochondria was determined on a TCA extract of the mitochondria after the mitochondria had been separated from the suspending medium by centrifugation. The colorimetric

magnesium method of Orange and Rhein (13) was modified in that 2.0 ml. of TCA were added to 1000 equivalent mg. of Mt, the resulting suspension was left in the refrigerator for 1 hour, and an aliquot taken for the determination in a smaller volume (3.0 ml.) than that described previously (13). The color development followed Beer's law up to a concentration of 0.4 μmole of Mg^{++} per 3 ml., which gave an optical density of 0.195 to 0.205.

ATPase activity in the mitochondria was determined by the methods described by Potter *et al.* (7). Mitochondrial adenylate kinase was determined by the method of Siekevitz and Potter (12). Inorganic phosphate was determined by a modified method of Lowry and Lopez (14, 12). Hexokinase was prepared from bakers' yeasts according to F. Loomis,[2] and it contained no ATPase or adenylate kinase activity. AMP, ADP, and ATP were obtained either as the potassium or sodium salts from the Pabst Laboratories, Milwaukee.

In the experiments to determine the intramitochondrial concentrations of compounds absorbing at 260 mμ, a modification of the method which has been worked out in this laboratory was used (15). The 12 equivalent gm. of Mt were separated from the incubation medium by centrifugation as described earlier. The mitochondria were taken up in 8.0 ml. of water, 4.0 ml. of 1.5 N PCA were added, and the resulting suspension was left in the refrigerator for at least 1 hour. After the denatured protein was spun down, the supernatant fluid was neutralized with 1.5 N KOH. The resulting suspension was left overnight in the refrigerator, and the insoluble potassium perchlorate was then centrifuged off. The resulting supernatant fluid was made just alkaline with NH_4OH and was placed on a Dowex 1 anion exchange column (200 to 400 mesh, 0.95 sq. cm. $\times$ 3 cm.). The column had been previously treated with 3 M HCOONa and with 7 N HCOOH to obtain the resin in the formate form, the excess formic acid being washed off with water. Approximately 95 per cent of the total E_{260} material contained in the extract was held on the column. Previously, when a Technicon automatic fraction collector and an apparatus designed to give an increasing concentration of HCOOH and of $HCOONH_4$ were used (15), the acid-soluble fraction from mitochondria exhibited thirteen peaks with absorption at 260 mμ. Repeated trials to duplicate this chromatogram manually yielded a chromatogram with a fairly good separation of the most prominent peaks. This chromatogram could be reproduced by consecutive 5.0 ml. elutions with known concentrations of either HCOOH or mixtures of HCOOH and $HCOONH_4$. The eluents are given with the data. The following nucleotides, AMP, ADP, ATP, DPN, TPN, IMP, ITP, were located by placing known samples of these on the column and by their absorption spectra. The peaks of these various compounds always

[2] Unpublished method.

came out at the same point, plus or minus one tube, in different experiments. The micromoles contained in each fraction were calculated by arbitrarily using 13,500 (the molecular extinction coefficient of the adenine moiety) after first subtracting the known blank reading due to the nonspecific absorption of the eluent. It is not implied that no other nucleotides are present, since rechromatography (15) has not been carried out.

Results

Release of E_{260} Material—It is especially significant that, under certain conditions of incubation, the acid-soluble nucleotides of rat liver mitochondria do not diffuse into the surrounding medium. This is especially true at 0°. Fig. 1 shows that no E_{260} material is released from the mitochondria at this temperature during a 60 minute incubation. In other experiments the E_{260} material was maintained for as long as 3 hours, and even after 5 and 10 hours at 0° the loss was negligible.

The remaining curves in Figs. 1 and 2 represent incubations at 30°. They show that at this temperature there is a progressive loss of E_{260} material in the presence of isotonic sucrose alone (control). Dilution of the mitochondria from 1000 equivalent mg. per 4 ml. to a volume of 34 ml. had no effect on the rate of E_{260} release.

Certain environmental conditions greatly modify this result. IP buffer (pH 7.2), at a final concentration of 0.01 M, as the sole addition to the sucrose medium, greatly accelerates the E_{260} release (Figs. 1 and 2),[3] but on lowering the phosphate concentration to 0.0025 M it had no effect. The addition of AMP, or AMP plus Mg^{++}, with the phosphate did not modify the results appreciably (Fig. 2). However, the addition of oxidizable substrate to the mixture with AMP, IP, and Mg^{++} slows and nearly stops the rate of loss, although it continues at a slow rate so that after 3 hours it becomes highly significant (not shown). Thus, only under those conditions in which there is an active uptake of IP (Fig. 2) is the E_{260} release slowed down at 30°. The addition of Mg^{++} is essential for maximal maintenance (Fig. 2), but, when Mg^{++} alone is added to the sucrose medium (Fig. 1), there is usually a slowing of the release for about the first 20 minutes, but after 60 to 90 minutes the release is equal to that of the control. This effect of Mg^{++} thus parallels its effect on the latent ATPase activity of resting mitochondria (7), and it is interpreted as a temporary maintenance of phosphorylative activity by the oxidation of endogenous substrates when none are added (Fig. 1) and a prolonged maintenance of phosphorylative activity when exogenous substrates are added (Fig. 2).

[3] The E_{260} material released by IP at 30° has the same absorption spectrum as that released at 30° in the presence of sucrose alone.

The results were also modified by non-physiological factors, including fluoride and DNP. Fluoride either greatly diminished (Fig. 1) or else had no effect on the extent of the early E_{260} release, but it always inhibited

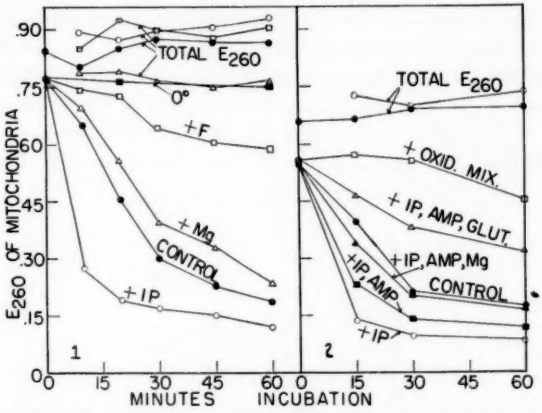


FIG. 1. E_{260} release of mitochondria incubated in sucrose; effects of F, Mg^{++}, and IP. Each tube contained 500 equivalent mg. of Mt, 2.6 ml. of isotonic sucrose solution, and, when added, 0.01 M F (0.01 M NaCl to the control), 0.003 M $MgCl_2$, and 0.01 M IP buffer (pH 7.2), the total volume in all cases being 4.0 ml. After the specified incubations at 0° or at 30°, the mitochondria were centrifuged, washed once with isotonic sucrose solution, taken up in 4.0 ml. of water, and 2.0 ml. of 1.5 N PCA were added to the mitochondrial suspension and to the medium. The acid extracts were then read at 260 mμ.

FIG. 2. Effect of oxidative phosphorylation on E_{260} release. Each tube contained 500 equivalent mg. of Mt in a total volume of 4.0 ml., the final solution being made isotonic with respect to 0.25 M sucrose solution. The oxidative mixture contained 0.01 M IP buffer (pH 7.2), 0.003 M $MgCl_2$, 30 μmoles of glutamate, and 3 μmoles of AMP. The mitochondria and the various media were incubated for varying lengths of time at 30° and then treated as described in Fig. 1. There was an appreciable IP uptake only in the tubes containing IP, AMP, and glutamate, and in those containing IP, AMP, Mg^{++}, and glutamate. The acid extracts of the mitochondria were prepared as in Fig. 1.

the release from 20 minutes on. The effects with DNP will be discussed later.

It had been found by previous investigators that the addition of ADP or ATP added stability to the oxidative ability of a homogenate (5), mitochondria (16), and of a heart muscle sarcosomal preparation (17). The effects on the E_{260} release of various levels of these nucleotides have been determined, but there seems to be no marked inhibitory effect on the release.

Intramitochondrial Magnesium—Fig. 3 illustrates the effects on the intramitochondrial Mg^{++} concentration when mitochondria were incubated at 30° in sucrose alone or in the presence of IP or of F. It was found that there is approximately 0.6 to 1.0 μmole of Mg^{++} per 1 equivalent gm. of Mt. The increase in the Mg^{++} concentration in the case of the control (Fig. 3), even above that for the zero time value, has been duplicated, except that this peak comes at various times of the incubation, from 5 to 20 minutes. It is believed the acid extraction of the mitochondria was complete, since, when larger amounts of mitochondria were used, they could be washed once again with acid, and this same peak was obtained,

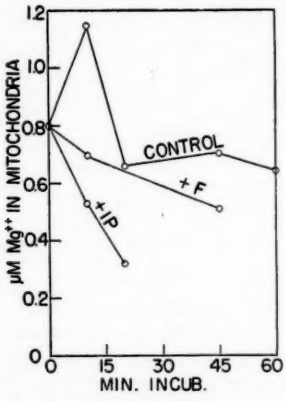


FIG. 3. Effects of preincubation of mitochondria with F or with IP on intramitochondrial Mg^{++}. Each tube contained 1000 equivalent mg. of Mt in a total volume of 3.0 ml. of isotonic sucrose. When added, F was at 0.015 M and IP at 0.01 M final concentration. Mg^{++} was determined as described in the text.

while it was also possible to determine fairly accurately the amount of Mg^{++} released into the medium. There was no increase in the Mg^{++} of the medium during the incubation of the control, even when the E_{260} release had increased 2-fold and when there was an increase in the intramitochondrial Mg^{++}. No explanation can be given at present for the anomalous intramitochondrial Mg^{++} curve. However, it is clear that at some point during the incubation the Mg^{++} is temporarily released from some bound site in the mitochondria, remains within the mitochondria, and is available for the test. The presence of 0.01 M F progressively lowers the intramitochondrial concentration of Mg^{++} (Fig. 3), indicating that either F competes with some binding site for Mg^{++} and the unbound Mg^{++} is then released into the medium, or more likely that, in the presence of mitochondria, F forms a complex with the Mg^{++}, making it

unavailable for the determination.[4] Paralleling its effect on the E_{260} material, incubation with IP decreases the concentration of intramitochondrial Mg^{++} (Fig. 3).

Nature of E_{260} Release—The absorption spectrum of the E_{260} release was compared to that of the material extracted from fresh mitochondria by PCA and found to be almost identical.[3] When compared to a known sample of ATP, the absorption spectra were also nearly identical in the

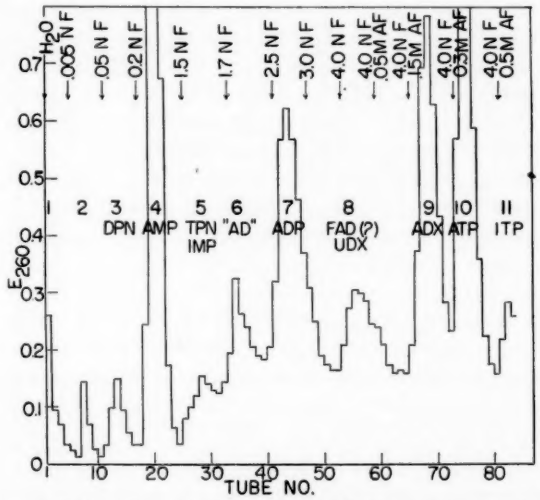

H2O
.005 N F
.05 N F
.02 N F
1.5 N F
1.7 N F
2.5 N F
3.0 N F
4.0 N F
4.0 N F .05M AF
4.0 N F .15M AF
4.0 N F .03M AF
4.0 N F .05M AF
1 2 3 4 5 6 7 8 9 10 11
DPN AMP TPN IMP "AD" ADP FAD(?) UDX ADX ATP ITP
E260
TUBE NO.

FIG. 4. Ion exchange chromatogram of acid-soluble compounds absorbing maximally at 260 mμ, obtained from rat liver mitochondria. The acid extracts of the mitochondria and the ion exchange column were prepared as described in the text. After the acid extract was placed on the column, successive 5.0 ml. aliquots of the eluents were put through. F = formic acid; AF = ammonium formate; AD, ADX = unknown nucleotides believed to contain adenine (15).

range of 250 to 270 mμ. It is clear that much of the release is made up of adenine nucleotides. However, it is recognized that compounds present in very small amounts may play an important rôle in the mitochondria. Based on the absorption coefficient of the adenine ring, there is approximately 0.4 μmole of acid-soluble nucleotides per 1 equivalent gm. of Mt. On the basis of these results, a quantity of mitochondria was prepared from 12 gm. of liver, and the ion exchange column was used to examine the nucleotides in greater detail. Fig. 4 shows the results of such a separa-

[4] F, IP, PP, or the adenine nucleotides, alone or in various combinations, had no effect on the color development in the Mg^{++} test.

tion with mitochondria from 12 gm. of rat liver. This chromatogram forms the basis for further studies on individual fractions to be reported later.

E_{260} Release and Oxidative Phosphorylation. Effects of Preincubation Conditions—The preceding sections have shown that mitochondria contain a significant amount of adenine nucleotides and that these substances are lost to the medium at various rates, depending on the temperature and the nature of the medium. Further studies were carried out on the relation-

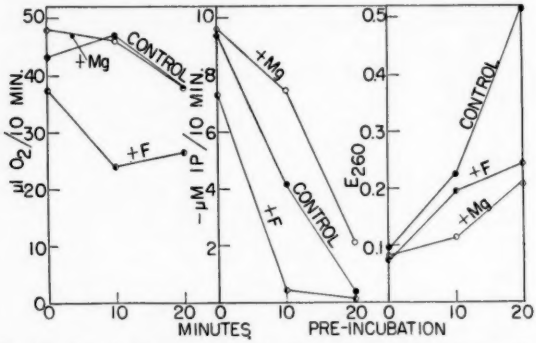


FIG. 5. Effects of preincubation of mitochondria with Mg^{++} or with F on oxidative phosphorylation and E_{260} release. 1200 equivalent mg. of Mt were incubated at 30° in the presence of fluoride or Mg^{++} with sufficient isotonic sucrose to give a final volume of 4.0 ml. At the times indicated, an aliquot containing 200 equivalent mg. of Mt was transferred to the oxidative phosphorylation test system, which contained 30 μmoles of IP buffer (pH 7.4), 60 μmoles of succinate, 12 μmoles of AMP, 1.3×10^{-5} M cytochrome *c*, 9 μmoles of $MgCl_2$, 22 μmoles of glucose, 0.1 ml. of hexokinase, and enough sucrose to make the solution isotonic in a final volume of 3.0 ml. Incubation at 30° for 10 minutes, with a 5 minute equilibration period. E_{260} release was measured as in Fig. 1.

ship between the loss of nucleotides and of magnesium with oxidative phosphorylation.

Fig. 5 presents the results of a typical experiment in which the effect of brief preincubation in sucrose alone (*control*) results in a loss of E_{260} material and of the capacity for oxidative phosphorylation, while the ability to oxidize succinate was essentially unaffected (*cf.* (16)). The presence of *magnesium* during the preincubation period delayed the initial output of nucleotides and concomitantly prolonged maintenance of oxidative phosphorylation. The effect of *fluoride* was to damage greatly oxidative phosphorylation *without* a corresponding effect on nucleotide loss. In all experiments with fluoride the final output of nucleotides was small (see Fig. 1) and the subsequently tested P:O ratios were very low, although

there was some variation in the rate of the initial output of nucleotides. In all cases there was a significant though small loss in nucleotides (see Figs. 1 and 3), and it is recognized that the lost material may be relatively more important for the phosphorylation system than the total nucleotide content. The effect of fluoride on phosphorylation cannot be attributed to a carry-over of fluoride into the final reaction mixture, since experiments with corresponding dilutions of fluoride (0.0015 M) did not inhibit phosphorylation in this system. The effect on oxygen uptake may reflect an action of fluoride plus phosphate on the succinate-oxidizing system (18). The effect on phosphorylation cannot be due to action on adenylate kinase (12), which was unaffected after preincubation with fluoride, because ADP in place of AMP was unable to yield a phosphorylative system. When mitochondria preincubated with fluoride were tested for ATPase activity (7), the activity was undiminished when the test system contained Ca^{++} plus Mg^{++}, but markedly diminished when only DNP was added.

When *inorganic phosphate* was added, the losses of Mg^{++} and nucleotides (Figs. 1 to 3) again correlated with the loss in oxidative phosphorylation, as in the case of the control and Mg^{++} preincubations. Similar effects of phosphate on nucleotide output and β-hydroxybutyrate oxidation have been observed by Hunter and Ford (19), and recent reports show decreases in intramitochondrial K^+ ((20), *cf.* (21)) and decreases in the ability to oxidize sorbate, malate, and α-ketoglutarate under the influence of inorganic phosphate (22). Preincubation with IP had no effect on adenylate kinase or on the DNP-activated (7) ATPase activity and slightly increased the ATPase response to the combination of Ca^{++} and Mg^{++}.

Inorganic pyrophosphate and arsenate (*cf.* (23, 24)) gave the same results that inorganic phosphate did at the same concentrations when tested for their effect on E_{260} release and on mitochondrial Mg^{++}. Preincubation with glutamate, succinate, or with hexokinase and glucose preserved oxidative phosphorylation compared with the effect of sucrose alone.

Effects of Preincubation with Mn^{++}—Although Mn^{++} stimulates many systems that are activated by Mg^{++}, it differs in certain respects from Mg^{++} (25–27) and has recently been suggested to have an important rôle in oxidative phosphorylation (10). Experiments of the type shown in Figs. 1 to 3 and 5 were carried out with various levels of Mn^{++} in the preincubation system, as shown in Fig. 6. It may be seen that the effects of preincubation in sucrose alone are almost completely ameliorated by low concentrations of Mn^{++}, *i.e.* 0.5 and 1.0×10^{-3} M, while higher concentrations of Mn^{++} cause losses in nucleotides and phosphorylating ability equal to or greater than preincubation in sucrose alone. At all the levels of Mn^{++} there was a partial loss in intramitochondrial Mg^{++} that was relatively unaffected by the Mn^{++} concentration. When the low levels of Mn^{++} were

added to the oxidative test system *directly*, they inhibited oxidative phosphorylation instead of preserving it as in the preincubation system.

Chromatographic Separation of Mitochondrial Nucleotides—Since preincubation in the presence of fluoride caused a decrease in the P:O ratio without much change in the E_{260} content of the mitochondria, it was decided to examine the intramitochondrial nucleotides in more detail, as an important change might have occurred in only one or two components out of the many that had been shown to occur (Fig. 4). Table I presents the results when mitochondria were preincubated under various conditions.[5]

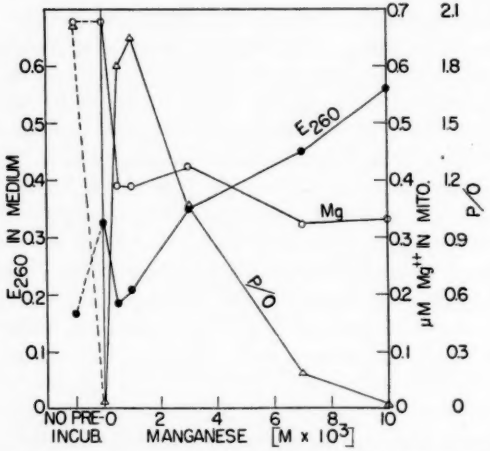


FIG. 6. Effect of preincubation of mitochondria with Mn^{++} on E_{260} release, oxidative phosphorylation, and intramitochondrial Mg^{++}. The flasks, which were preincubated for 15 minutes at 30°, contained 1750 equivalent mg. of Mt and the given concentrations of $MnCl_2$ in a total volume of 4 ml. of isotonic sucrose solution. After the preincubation, aliquots containing 175 equivalent mg. of Mt were taken for the determination of oxidative phosphorylation. The rest of the mitochondrial suspension was centrifuged and the E_{260} release and intramitochondrial Mg^{++} concentration were determined. The oxidative phosphorylation medium was the same as in Fig. 5, and the approximate P:O ratio was determined by extrapolating the oxygen uptake to include the equilibration time. When the mitochondria were not preincubated, no manganous ions were present and the mitochondria were taken out immediately for the separate determinations. The dash lines represent preincubation in sucrose alone.

[5] In various experiments with this same amount of mitochondria, there are approximately 1 μmole of ADP and ATP, slightly more AMP, and 1 to 2 μmoles of IP in the acid extract. The IP is also released from the mitochondria by incubating at 30° for 20 minutes. In a comparison of the nucleotides contained in each of the

Since different batches of mitochondria were used in the experiments, the amounts of nucleotide in the various main fractions are reported as percentages of the total absorption at 260 mμ. It can be seen that under

TABLE I

Effects of Preincubation with Various Factors on Oxidative Phosphorylation and on Nucleotide Concentration in Rat Liver Mitochondria

Each 30° preincubation flask contained a different batch of 12 equivalent gm. of Mt in a total volume of 36 ml. of isotonic sucrose solution, and, when added, 0.003 M Mg^{++}, 0.01 M F, and 0.01 M glutamate. The phosphorylation test system contained 333 equivalent mg. of Mt, 30 μmoles of IP buffer, pH 7.2, 60 μmoles of succinate, 12 μmoles of AMP, 22 μmoles of glucose, 1.3×10^{-5} M cytochrome *c*, 0.003 M $MgCl_2$, 0.1 ml. of hexokinase, enough solid sucrose to make a final isotonic solution, and enough isotonic sucrose solution to make a total volume of 3.0 ml. Incubation at 30° for 15 minutes. The mitochondria for the phosphorylation test were obtained from the larger suspensions of mitochondria before and after preincubation under the various conditions listed. IP uptake was determined as described in the text. The intramitochondrial nucleotide concentrations were obtained as described in the text and in Fig. 4.

	No preincubation	Preincubation additions			
		None	Mg^{++}	F	Glutamate
	μmoles IP uptake per 15 min.				
0 min. preincubation	13.3	13.3	12.2	10.5	8.0
20 " "		12.3	11.4	1.0	8.1
	Per cent of total E_{260} after preincubation				
Peak 1.	2.4	2.1	4.3	3.0	2.8
" 2.	1.3	1.4	1.8	0.6	0.9
" 3. DPN	1.8	2.0	2.5	3.4	2.8
" 4. AMP	17.6	17.4	16.4	21.9	15.7
" 5. TPN, IMP	3.4	3.3	3.5	6.0	2.6
" 6.	6.7	7.3	6.9	6.1	6.1
" 7. ADP	20.5	19.7	19.1	19.6	17.3
" 8. FAD, UDX	10.6	9.6	10.7	12.9	11.0
" 9.	17.9	17.8	17.6	12.7	18.1
" 10. ATP	17.7	19.6	17.2	13.8	22.7

conditions of active phosphorylation, that is, in all cases except the fluoride preincubation, the percentages in the various peaks were remarkably con-

peaks in the mitochondria compared to that found in the whole cell, it was found that the amounts of AMP, ADP, ATP, TPN, and Peaks 6 and 9 in the mitochondria were approximately 5 per cent of the amount in the whole cell. Peaks 1, 2, and 8 were in concentrations approximately 15 per cent of those in the whole cell, while DPN was in a concentration approximately 1 per cent of that in the whole cell.

stant. When fluoride had been present, there was a significant decrease in only two of the fractions, ATP and Peak 9 which has been referred to elsewhere as Ad-X (15) because it is an unknown adenosine nucleotide. The function and structure of the constituent compound (or compounds) are unknown, but it has been shown to have very low radioactivity compared to ADP and ATP when inorganic P^{32} is given either *in vivo* (Brumm, Siekevitz, and Potter, unpublished) or *in vitro* (9).

It was mentioned earlier that the addition of hexokinase and glucose during preincubation stabilized oxidative phosphorylation. When such mitochondria were examined chromatographically, the percentage of nucleotides present as Peak 9 was markedly increased, which is in harmony with the observation that when oxidative phosphorylation was decreased the concentration was lower.

Experiments with DNP were somewhat surprising in that there was either no change or a decrease in the amount of E_{260} material coming out of the mitochondria at 30°.[6] This finding is in contrast to a report by Dresel (28) who used a different type of preparation. Chromatographic examination of the intramitochondrial nucleotides showed that DNP (3×10^{-5} M) increased the AMP concentration at the expense of ATP, while ADP and Ad-X were essentially unchanged. This effect of DNP probably reflects actions of DNP on ATPase activity (6, 7) in the presence of adenylate kinase (12) and on the intramitochondrial condition for subsequent changes in the medium (8).

DISCUSSION

The fact that acid extracts of many batches of both fresh mitochondria and mitochondria obtained after oxidation *in vitro* have shown a constancy in the concentrations of the individual nucleotides is indicative that these mitochondrial nucleotides are not freely diffusible from the mitochondria, and that the mitochondria have a limited ability to retain nucleotides that is conditioned by their environment and resulting physiological state. It is now known that these intramitochondrial nucleotides are not in complete equilibrium with the nucleotides added to the medium (8, 9), that they are as readily, or even more, available to the mitochondrial enzymes as the nucleotides in the medium seem to be ((8, 9), *cf.* (29)), and that the sites at which the nucleotides are localized or bound are probably also the sites where oxidative phosphorylation takes place (9).

It is not clear whether the phosphorylation mechanism is destroyed because of the unbinding of the nucleotides, Mg^{++}, or K^{++} (20, 30, 31) from some active site, or because these cofactors are simply lost from the

[6] Since DNP itself absorbs at 260 mμ, the amount of the absorption due to DNP had to be subtracted from that of the total to obtain that due to the purine ring.

mitochondria.[7] However, the results of preincubation with fluoride and the fact that fluoride can form complexes with Mg^{++} and phosphate groups would tend to favor the former reason.[8] Therefore, it may be that the action of fluoride is to immobilize these cofactors of phosphorylation at their site of activity, while the action of IP and of high concentrations of Mn^{++} is to release them from this active site, each process making them unavailable for participation in the phosphorylation mechanism. It is still possible that the nucleotides are bound to proteins enzymatically active in oxidative phosphorylation, conferring stability upon these enzymes, and that the unbinding of the nucleotides makes the proteins more labile as well as enzymatically inactive.

The present studies show the presence of a variety of known and unknown nucleotides within the mitochondria and that their distribution is not the same as that of the whole liver. The studies further support the idea that the mitochondrion is an organized unit of life that requires much further study in terms of intramitochondrial events and their relation to the rest of the cell. From the experimental standpoint we can add the intramitochondrial nucleotide balance to the P:O ratio and the latency of ATPase activity and oxidative rate and capacity (7, 8, 32) as experimental criteria for judging the physiological state of the mitochondria. Finally, the need for correlations between these criteria and the morphological and physical studies of others (33–36) needs to be constantly borne in mind.

SUMMARY

1. Intramitochondrial concentrations of compounds absorbing maximally at 260 mμ and of Mg^{++}, and the loss of these compounds from the mitochondria, were noted under various experimental conditions.

2. Mitochondria incubated in sucrose at 30° quickly lose their acid-soluble nucleotides and their ability to couple phosphorylation to oxidation. Loss of these nucleotides can be prevented by conditions which promote or maintain oxidative phosphorylation by the mitochondria.

3. It has been noted that inorganic phosphate and high concentrations of Mn^{++} catalyze the loss of phosphorylative ability (lower the P:O ratio) at the same time that they catalyze the loss of adenine nucleotides. Low concentrations of Mn^{++} prevent both the loss of oxidative phosphorylation and the loss of E_{260} material. Compounds like inorganic pyrophosphate and arsenate also catalyze the release of nucleotides from the mitochondria.

[7] In an attempt to restore oxidative phosphorylation in mitochondria preincubated with IP, it was found that even when the IP-generated E_{260} release, including protein, from 10 equivalent gm. of Mt was returned to 200 equivalent mg. of inactivated Mt there was no restoration (experiments performed with the aid of Dr. Y. Takagi).

[8] It is indicative that F has been found to inhibit mitochondrial adenylate kinase in a non-competitive manner (unpublished experiments of Siekevitz and Potter).

4. If mitochondria are incubated in the presence of F, they become inactive with respect to phosphorylation, without losing their nucleotide or Mg^{++} content. From various experiments it was inferred that F binds some compound or compounds necessary for mitochondrial phosphorylation.

5. Ion exchange chromatograms of the acid-soluble material absorbing at 260 mμ have been performed after the mitochondria have been under various treatments leading to inactive or active phosphorylation. Under conditions of inactive phosphorylation, as induced by pretreatment with F, only ATP and an unknown adenine nucleotide are reduced in concentration within the mitochondria.

BIBLIOGRAPHY

1. Siekevitz, P., and Potter, V. R., *Federation Proc.*, **12,** 267 (1953).
2. Potter, V. R., *Arch. Biochem.*, **6,** 439 (1945).
3. Hunter, F. E., Jr., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 297 (1951).
4. Lehninger, A. L., *J. Biol. Chem.*, **178,** 625 (1949).
5. Pardee, A. B., and Potter, V. R., *J. Biol. Chem.*, **181,** 739 (1949).
6. Potter, V. R., and Recknagel, R. O., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 377 (1951).
7. Potter, V. R., Siekevitz, P., and Simonson, H. C., *J. Biol. Chem.*, **205,** 893 (1953).
8. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **201,** 1 (1953).
9. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **215,** 237 (1955).
10. Lindberg, O., and Ernster, L., *Nature*, **173,** 1038 (1954).
11. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183,** 123 (1950).
12. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **200,** 187 (1953).
13. Orange, M., and Rhein, H. C., *J. Biol. Chem.*, **189,** 379 (1951).
14. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162,** 421 (1946).
15. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209,** 23 (1954).
16. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191,** 485 (1951).
17. Slater, E. C., and Cleland, K. W., *Biochem. J.*, **55,** 566 (1953).
18. Slater, E. C., and Bonner, W. O., Jr., *Biochem. J.*, **52,** 185 (1952).
19. Hunter, F. E., Jr., and Ford, L., *Federation Proc.*, **13,** 233 (1954).
20. Stanbury, S. W., and Mudge, G. H., *Proc. Soc. Exp. Biol. and Med.*, **82,** 675 (1953).
21. Pressman, B. C., and Lardy, H. A., *J. Biol. Chem.*, **197,** 547 (1952).
22. Witter, R. F., and Newcomb, E., *Federation Proc.*, **11,** 313 (1952).
23. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **170,** 147 (1947).
24. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365 (1951).
25. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1942).
26. Utter, M. F., and Werkman, C. H., *J. Biol. Chem.*, **146,** 289 (1942).
27. Bentley, O. G., and Phillips, P. H., *Arch. Biochem. and Biophys.*, **32,** 338 (1951).
28. Dresel, E. I. B., *Biochem. J.*, **54,** 654 (1953).
29. Slater, E. C., and Holton, F. A., *Biochem. J.*, **55,** 530 (1953).
30. Bartley, W., and Davies, R. E., *Biochem. J.*, **52,** p. xx (1952).
31. MacFarlane, M. G., and Spencer, A. G., *Biochem. J.*, **54,** 569 (1953).

32. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195**, 215 (1952).
33. Harman, J. W., *Exp. Cell Res.*, **1**, 394 (1950).
34. Harman, J. W., and Feigelson, M., *Exp. Cell Res.*, **3**, 47, 509 (1952).
35. Raaflaub, J., *Helv. physiol. et pharmacol. acta*, **11**, 142, 157 (1953).
36. Schneider, W. C., *J. Histochem. and Cytochem.*, **1**, 212 (1953).

BIOCHEMICAL STRUCTURE OF MITOCHONDRIA

II. RADIOACTIVE LABELING OF INTRAMITOCHONDRIAL NUCLEOTIDES DURING OXIDATIVE PHOSPHORYLATION*

By PHILIP SIEKEVITZ† and VAN R. POTTER

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisconsin)

(Received for publication, December 21, 1954)

In a recent paper (2) we postulated that during oxidative phosphorylation by isolated mitochondria the turnover rates of the adenine nucleotides within the mitochondria are much more rapid than their passage in and out of the mitochondria. This paper presents evidence to confirm that hypothesis by using radioactivity measurements as a more direct method of determining turnover and diffusion. The data also suggest that the mitochondrial enzyme, adenylate kinase (3–5), exists in two sites in the mitochondrion. Based on these results, a scheme is presented for certain aspects of the biochemical structure of the mitochondrion, and the possible rôle of such a structure in regulating oxidation is discussed.

EXPERIMENTAL

The rat liver mitochondria were prepared as described previously (6). In all experiments the mitochondria from 12 gm. wet weight of rat liver were used (12 equivalent gm. of Mt). In order to stop the enzymatic reactions before equilibrium was attained, the cold isotonic sucrose suspension of mitochondria was mixed with the cold oxidative medium (components given in Tables I to VI, total volume usually 26 ml.), and, immediately after mixing, the mitochondria were separated from the medium by centrifugation for 10 minutes at 7500 × *g* in the International refrigerated centrifuge kept at 0°. (For convenience this type of experiment is henceforth called centrifugation-incubation.) The mitochondria were washed once with cold isotonic sucrose and separated again as described above. The mitochondrial pellet was taken up in 8 ml. of cold water, 4 ml. of cold 1.5 N perchloric acid were added, and the suspension was left in the refrig-

* This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and in part by an institutional research grant (No. 71) from the American Cancer Society. A part of this paper was presented at the meeting of the American Society of Biological Chemists at Atlantic City, April 16, 1954 (1).

† Present address, Laboratories of The Rockefeller Institute for Medical Research, New York.

XUM

erator for 1 hour. After centrifuging the denatured protein, the precipitate was washed once with water, the washings were combined with the initial supernatant solution, and the whole was neutralized with 1.5 N KOH and left in the refrigerator overnight. After separation of the mitochondria from the oxidative medium, a one-tenth aliquot was taken from the medium, and an acid extract of the medium was prepared as described for the acid extract of the mitochondria. In both cases, the insoluble $KClO_4$ was centrifuged and the supernatant fluid was neutralized in preparation for ion exchange chromatography.

The Dowex 1 ion exchange columns were set up in glass tubing of 1.1 cm. diameter by 15 cm. long, sealed to the bottom of a 125 ml. Erlenmeyer flask. Their preparation and the placement of the neutralized extracts on the columns were as described previously (6). The successive 5.0 ml. eluents which were used for the consecutive elution of the various nucleotides are given in Fig. 1. The various nucleotides were located by placing samples of them on the columns, by a comparison of placement on similar columns described earlier (7), by their absorption spectra, and, in the case of the adenine and inosine phosphates, by the ratios of purine to stable phosphate to labile phosphate. Inorganic phosphate and glucose-6-phosphate were located by comparison with known samples and by determination of inorganic phosphate (Soyenkoff method (8)) and by the anthrone test (9). The presence of a flavin adenine dinucleotide was determined on the column by the coincidence of the peak of the yellow color (maximum at 445 mμ) with that of the ultraviolet peak at 260 mμ. In the columns described previously (6), in which elution was begun with formic acid, IP[1] and G6P appeared just before the ADP peak. In order to effect a clear-cut separation of these compounds, 0.1 M ammonium formate was put through the column initially. This low concentration of ammonium formate had the effect of moving IP and G6P ahead on the chromatogram, the other compounds remaining stationary (Fig. 1; also Khym and Cohn (10)). When necessary, the IP and G6P could be separated from each other by using 0.04 M ammonium formate as the initial eluent, in which case the G6P was eluted sufficiently before the IP, and thus accurate specific activities could be obtained for both compounds. In the case of the acid extract from the medium, a simplified elution procedure was used, since the added adenine nucleotides were much greater in quantity than any other nucleotides which might be present. In this case, only the solu-

[1] Abbreviations are used as follows: AMP, ADP, ATP = adenosine mono-, di-, and triphosphate; DPN and TPN = diphospho- and triphosphopyridine nucleotide; IP = inorganic orthophosphate; G6P = glucose-6-phosphate; CMP, GMP, and IMP = cytosine, guanosine, and inosine monophosphates; FAD = flavin adenine dinucleotide; ITP = inosine triphosphate; DNP = 2,4-dinitrophenol; UDX = derivative of uridine diphosphate; Mt = mitochondria.

tions for separating IP, AMP, ADP, and ATP (see Fig. 1) were put through the column. The micromoles contained in each tube were calculated by arbitrarily using 13,500 as the molecular extinction coefficient (at 260 mμ) of the adenine moiety, after first subtracting the known blank reading due to the non-specific absorption of the eluent.

Each fraction from the chromatogram was counted directly by means of a Geiger-Müller dip counter (obtained from Tracerlab, Inc., Boston)

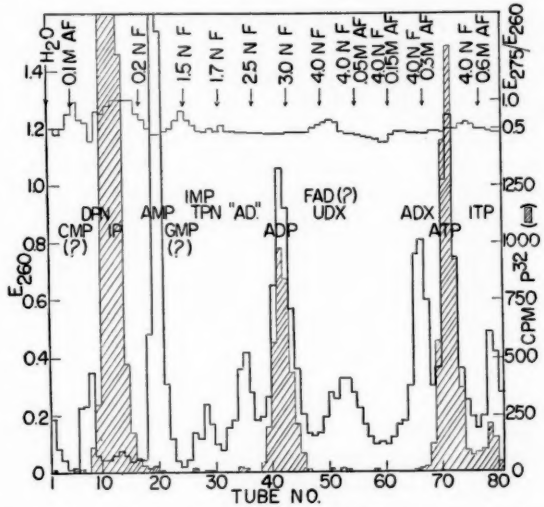


FIG. 1. Ion exchange chromatogram of acid-soluble compounds absorbing maximally at 260 mμ obtained from rat liver mitochondria after undergoing oxidative phosphorylation in the presence of IP^{32}. See "Experimental" for the procedure. F = formic acid; AF = ammonium formate; AD, ADX = unknown nucleotides believed to contain adenine (7).

and a Berkeley scaler. Corrections were made for the radioactivity decay. The carrier-free radioactive IP^{32} was obtained from the Atomic Energy Commission, Oak Ridge, Tennessee.[2] In one experiment all the fraction tubes from the column were counted, and there was no radioactivity other than when phosphate-containing compounds were found.

Fig. 1 gives a typical chromatogram of the acid extract of mitochondria after oxidative phosphorylation had taken place in the presence of IP^{32}. It can be seen that in ADP and ATP the radioactivity follows very closely the E_{260} absorption. In previous experiments with formic acid as the

[2] The IP^{32} was allocated to Dr. Charles Heidelberger of this laboratory, to whom we are indebted for advice on the use of the counters.

initial eluent (see above), the AMP and DPN were entirely free of any IP, and it was found that these two compounds were completely non-radioactive. Also, the Ad-X could be more easily separated from ATP and it contained no radioactivity. The specific activity of each compound was determined by averaging those of from three to five of the peak tubes; the specific activities of the individual tube contents rarely differing by more than ±10 per cent of the average specific activity. In some cases, ADP and ATP were rechromatographed on an ammonium formate column (7), and the specific activity thus obtained agreed closely with that obtained initially.

Hexokinase was prepared from bakers' yeast according to F. Loomis,[3] and it contained no adenylate kinase or ATPase activities, but did contain inorganic pyrophosphatase activity. AMP and ATP were obtained either as the sodium or potassium salts from the Pabst Laboratories, Milwaukee. In the experiments utilizing ADP^{32}, the radioactive ADP was made during oxidative phosphorylation by mitochondria utilizing IP^{32}. The ADP^{32} was obtained by ion exchange chromatography by using the initial washing with ammonium formate to get rid of all contaminating IP^{32} (see above). The eluted ADP^{32} solution was lyophilized and reprecipitated through the barium salt.

Results

Effect of Added Nucleotides on Quantities and Specific Activities of Intra- and Extramitochondrial Nucleotides—When mitochondria were incubated at 25° for as little as 5 minutes with IP^{32} and either AMP or ADP in the oxidative medium, and then separated from the medium, it was found that the specific activities of the intramitochondrial ADP and ATP were the same as those in the medium. Thus in these experiments, in which 70 to 120 μmoles of IP were taken up, an equilibrium between the intra- and extramitochondrial nucleotides was attained. In order to observe whether there was any change in the concentrations of the intramitochondrial nucleotides during oxidative phosphorylation, a centrifugation-incubation experiment was set up (see "Experimental") in which incubation took place at 0° while the mitochondria were being separated from the medium by centrifugation. It was found that under these conditions IP^{32} was taken up from the medium, ADP^{32} and ATP^{32} were formed, and a net synthesis of ATP occurred. That oxidative phosphorylation can be shown to occur at 0° is probably due to the large concentration of mitochondrial enzyme used (*cf.* (12)). In these centrifugation-incubation experiments the IP uptake was reduced to a value of 10 to 35 μmoles, and, under these

[3] Unpublished method. This preparation corresponds to Fraction 3a of Berger *et al.* (11).

conditions of lowered phosphorylation, differences were observed between the specific activities of the intra- and extramitochondrial nucleotides, depending upon whether AMP or ADP was added to the medium. All the following results refer to centrifugation-incubation experiments.

Table I shows that the specific activity of the ATP (per micromole of adenine) was always nearly twice that of the ADP. During the prepara-

TABLE I

Comparison of Specific Activities of Intra- and Extramitochondrial Nucleotides When AMP or ADP Was Added Initially to Medium; Effect of KCN

In Experiment 1, each oxidative medium contained 200 μmoles of glutamate, approximately 60 μmoles of AMP or approximately 30 μmoles of AMP plus approximately 30 μmoles of ADP, 100 μmoles of IP, 63 μmoles of $MgCl_2$, 280 mg. of sucrose, and 0.2 ml. of IP^{32} containing approximately 250,000 c.p.m. The contents of the medium were the same in Experiment 2, except that 60 μmoles of ADP, or approximately 30 μmoles of AMP plus approximately 30 μmoles of ADP, or approximately 60 μmoles of AMP plus KCN at final concentration of 0.001 M were added in the appropriate flasks. Mixing of the mitochondria and media and the preparation of the acid extracts of mitochondria and media as described in the text. The specific activity in this and subsequent tables is given as P^{32} per micromole of purine as determined by absorption at 260 mμ.

Experiment No.	Additions	IP uptake, μmoles	IP*		ADP*		ATP*		Intramitochondrial nucleotides, μmoles			
			Intramitochondrial	Extramitochondrial	Intramitochondrial	Extramitochondrial	Intramitochondrial	Extramitochondrial	AMP	ADP	ATP	Ad-X
1	AMP		2290	2350	1980	2120	3650	3840				
	" ADP		2280	2430	1570	780	2790	1770				
2	ADP	13.4	2980	3830	2560	520	4320	1080	1.50	1.07	0.99	0.93
	" AMP	9.8	2950	3970	2830	430	4320	1130	1.50	1.52	1.21	0.95
	" KCN	0.5	2420		1960		2450		1.95	0.79	0.56	0.59

* Specific activity.

tion of various samples of radioactive ATP for rechromatography, some of the ATP broke down to ADP and IP, and it was found that the radioactive phosphates of ADP and the IP derived from the terminal P of ATP were approximately equal in specific activity. The results thus suggest that the adenylate kinase in these mitochondria is so highly active that the two high energy phosphates in ATP appear to react at the same rate (see also Krebs *et al.* (13)). When AMP was the initial nucleotide, very little difference was observed between the specific activities of the intra- and extramitochondrial ADP or ATP (Table I). When ADP was added, alone or with AMP, the specific activity of the ADP in the medium was less than that of the intramitochondrial ADP, as a result of the dilution of the

extramitochondrial ADP by the added ADP. However, the ATP which was synthesized was also lower in specific activity in the medium than in the mitochondria. This lowered specific activity is considered to be due to a dilution of the radioactive ATP formed during oxidative phosphorylation by non-radioactive ATP formed by some other process, these two sources of ATP not contributing their ATP to an immediately common pool. The results obtained by Cohn (14), using IP with labeled O^{18} and adding unlabeled ATP, are suggestive of the same conclusions. Since the two radioactive phosphates of ATP were approximately equal in specific activity, it would appear that the ATP was being diluted as a whole.

To be noted from Table I is that 0.001 M KCN reduced the specific activity of the intramitochondrial ATP more than that of the intramitochondrial ADP, probably because the residual oxidative phosphorylation was enough to turn over all of the labile phosphate in ADP and only a part of the phosphates in ATP. The results with KCN would indicate that most of the radioactivity in the ATP is not due to exchange reactions between IP and ATP, as described by Boyer *et al.* (15).[4] It can also be seen that KCN also greatly reduced the mitochondrial concentrations of ADP, ATP, and Ad-X, while it increased that of AMP, indicating that these mitochondrially bound nucleotides are probably generated by oxidative phosphorylation (6).

Table II presents a comparison of the specific activities obtained when AMP or ADP or AMP plus ATP was added initially. The results with AMP and ADP are similar to those of Table I. When AMP plus ATP was added, not only was the specific activity of the extramitochondrial ATP less than that of the intramitochondrial ATP (through dilution by the added ATP), but the specific activity of the extramitochondrial ADP was also less than that of the intramitochondrial ADP. This result is probably due to the fact that there is another source of ADP (non-radioactive) which dilutes the radioactive ADP formed through oxidative phosphorylation plus adenylate kinase action and which is in a relatively slow equilibration with it. The only known mitochondrial reaction which could produce non-radioactive ADP when AMP plus ATP is added, and non-radioactive ATP when ADP is added, is the mitochondrial adenylate kinase reaction (3–5). This suggests that when ADP is added it probably reacts immediately with a surface adenylate kinase to form AMP and non-radioactive ATP. The AMP goes into the mitochondria, to be acted upon by the interior adenylate kinase and through oxidative phosphorylation to form radioactive ADP and ATP. Under the conditions of the experiment, the rates of these reactions are higher than the rates of passage

[4] Actually, for the purpose of these experiments, it would not matter how the radioactivity got into the ATP, as long as it was an enzymatic reaction.

of the radioactive nucleotides out of the mitochondria, thus accounting for the differences in specific activities between the intra- and extramitochondrial nucleotides. The same explanation can be given when AMP plus ATP is added initially.

It can be seen from Tables I and II (and found in all cases) that when ADP was added the specific activity of the extramitochondrial ATP was always greater than twice that of the extramitochondrial ADP. This result may be further proof of the theory outlined above, for, while the radioactive ADP is being diluted only by non-radioactive ADP, the radioactive ATP is diluted by ATP produced by the surface adenylate kinase,

TABLE II

Comparison of Quantities and Specific Activities of Intra- and Extramitochondrial Nucleotides When AMP or ADP or AMP Plus ATP Is Added to Medium

Experimental conditions as for Table I. When used, approximately 60 μmoles of AMP or approximately 60 μmoles of ADP or approximately 40 μmoles of AMP plus approximately 40 μmoles of ATP were added. The theoretical IP uptake (see Table IV) was 33.4 μmoles with AMP, 25.5 μmoles with ADP, and 17.1 μmoles with AMP plus ATP. S.a. = specific activity.

Additions		IP	ADP		ATP		AMP
		S.a.	S.a.	μmoles	S.a.	μmoles	μmoles
AMP	Intramitochondrial	7140	5710		12,200		
	Extramitochondrial	8900	6350	17.0	16,020	8.2	35.8
ADP	Intramitochondrial	5810	4280		9,190		
	Extramitochondrial	7930	2550	15.9	5,800	34.7	9.2
AMP + ATP	Intramitochondrial	5350	4030		8,020		
	Extramitochondrial	7680	1650	25.1	4,320	37.1	20.1

this ATP being generated from both non-radioactive ADP and from radioactive ADP. Thus the radioactivity in the extramitochondrial ATP phosphates would always be greater than the radioactivity of the ADP phosphates.

Effect of Added ADP^{32} on Specific Activities of Intra- and Extramitochondrial Nucleotides—If the foregoing hypothesis is correct, it must be that when ADP is added the surface adenylate kinase reaction converts the ADP to AMP very rapidly, and it is AMP which goes into the mitochondrial interior to be utilized in oxidative phosphorylation. This can be demonstrated by using ADP^{32} (Table III). It can be seen from Table III that the specific activity of the intramitochondrial ADP and ATP is much less than that of the extramitochondrial nucleotides, while the specific activity of the intramitochondrial IP is much higher than that of the extramitochondrial IP. Thus it can be postulated that the added ADP^{32} gives

rise to ATP^{32} and non-radioactive AMP via the surface adenylate kinase; some of the ATP^{32} enters the mitochondria, mixes with the mitochondrial ATP, and is broken down to IP^{32} and ADP^{32}. These become involved in mitochondrial oxidative phosphorylation, and an equilibrium is quickly established between mitochondrial ADP, ATP, and IP. The breakdown of ATP to ADP and IP is inferred to have occurred at the same general site of oxidative phosphorylation. Thus it can also be suggested that the rate of passage of ADP and ATP *into* (as well as out of) the mitochondria is much less than the rate of interconversion of these nucleotides within the mitochondria. Also, since in this experiment there was a net oxidative synthesis of ATP, it must be that AMP was the form of the adenine nucleotide which entered the mitochondria to be utilized in this ATP synthesis.

TABLE III

Comparison of Specific Activities of Intra- and Extramitochondrial Nucleotides When ADP^{32} Is Added to Medium

Experimental conditions as for Table I, except that approximately 30 μmoles of ADP^{32}, containing approximately 500,000 c.p.m. of P^{32} and 100 μmoles of non-radioactive IP, were added. The calculated IP uptake was 10.0 μmoles.

Determined	Intramitochondrial, s.a.	Extramitochondrial	
		S.a.	μmoles
IP	1880	350	
AMP	0	0	5.2
ADP	2070	6,400	10.8
ATP	4130	12,680	15.2

Comparison of Calculated with Actual Dilutions of Nucleotide Radioactivity—Another way of explaining the results, when ADP is added together with IP^{32} in an oxidative system, is to say that the amount of dilution of the radioactivity in the ATP of the medium is a function of the amount of ATP which was synthesized by oxidative phosphorylation within the mitochondria compared to that produced by adenylate kinase action at some other mitochondrial site. The procedure for the calculation of the theoretical results and a comparison of the calculated and actual results are given in Table IV. The agreement is very good, even when AMP plus ATP is added and the radioactivity of the ADP of the medium is diluted.

Validity of Centrifugation-Incubation Method—The interpretations mentioned in the preceding paragraphs are based on the assumption that the method of incubation (incubating while separating the mitochondria from the medium at 0°) does not introduce an artifact into the results. From a theoretical standpoint, the measurements on the intramitochondrial nu-

cleotides can be regarded as yielding data that are the resultant of processes occurring (*a*) while the mitochondria are being spun down with opportunity to equilibrate with the medium (see below), and (*b*) while they are tightly packed in the pellet and lacking such opportunity (see below). During period (*b*) the differences in passage of compounds in and out of the mitochondria will be suppressed, but during period (*a*) such differences are expressible. Since the data represent the sum of differences during (*a*) and (*b*), differences between compounds should be detectable. Using

TABLE IV

Comparison of Observed with Calculated Dilution of Radioactivity of Extramitochondrial Nucleotides

Experiments performed as for Table I with added ADP only, except when noted below. The theoretical dilution was obtained as follows. When ADP was added, the amount of ATP formed in the medium was the resultant of two reactions: adenylate kinase (ADP → AMP + ATP), and oxidative phosphorylation (ADP → ATP). Therefore, by subtracting the amount of AMP in the medium at the end of the experiment from the amount of ATP in the medium, the ATP obtained via oxidative phosphorylation could be approximated.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11
$\frac{100 \times \text{S.a. extramitochondrial ATP}}{\text{S.a. intramitochondrial ATP}}$	25	73	58	53	50	63	56	59	26	26	41*
$\frac{100 \times \text{ATP by oxidative phosphorylation}}{\text{Total ATP}}$	31	78	57	63	42	73	58	59	30	26	40

* This experiment was with added AMP plus ATP; hence that figure = 100 × (s.a. of extramitochondrial ADP)/(s.a. of intramitochondrial ADP).

the same reasoning, Bartley and Davies (16), in their successful attempt to show concentration gradients between mitochondria and medium, employed centrifugation methods in which a large gravitational force was reached in a very short time and kept for a very short time in order to minimize process (*b*). In their process (*a*), these authors incubated at 20°, while in our process (*a*) we could, owing to the very large amounts of mitochondria used, incubate at 0° during the centrifugation.

A second type of possible artifact might arise if there were two or more kinds of mitochondria (17–19) in our preparation, the lighter ones containing relatively more adenylate kinase activity than the heavier ones. In this case an apparent separation of adenylate kinase activity from oxidative phosphorylation might occur. However, a variety of experiments

indicated that during the incubation-centrifugation of our mitochondrial preparation both adenylate kinase and oxidative phosphorylation activities came down together, and, moreover, the final supernatant medium contained no determinable adenylate kinase activity.

Intramitochondrial Dilution of Exogenous Inorganic Phosphate—In all experiments except one it was found that the specific activity of the intramitochondrial IP was less (sometimes by as much as 40 per cent) than that of the medium IP, confirming Crane and Lipmann (20) and Bartley and Davies (16). However, when AMP was added initially (20), the specific activities of the intra- and extramitochondrial ADP were approximately equal, and the specific activity of the ADP was the same as that of the intramitochondrial IP, in conformity with results with brain mitochondria (21), but not the same as that of the extramitochondrial IP, as was found previously (20). It would appear that there is a dilution of the added IP^{32} by non-radioactive mitochondrial phosphate, and it is this pool of phosphate which is tapped preferentially during oxidative phosphorylation. It has been found that in the 12 equivalent gm. of Mt used there was 1 to 2 μmoles of inorganic orthophosphate present initially.[5] Considering that in some experiments more than 17 μmoles of ATP were synthesized via oxidative phosphorylation and at the same time there is a dilution of some 50 per cent in the specific activity of the intramitochondrial IP, this small amount of mitochondrial inorganic orthophosphate could not account for the large continuing dilution observed.[6] We also do not think that ATP is a source of this phosphate, as elsewhere postulated (16), since, when AMP is the initial nucleotide, the specific activities of extra- and intramitochondrial ATP are equal, no non-radioactive ATP is formed, and there still exists the intramitochondrial dilution of the IP. The only large sources of mitochondrial phosphate able to account for the dilution are the phospholipide and phosphoprotein fractions (22, 23), but the rôle of these fractions has not been studied in detail.[7]

[5] After oxidative phosphorylation had taken place, the inorganic orthophosphate content of the mitochondria rose to 4 to 8 μmoles per 12 equivalent gm. of Mt. Thus the mitochondria can apparently increase their IP concentration (16), in contrast to the adenine nucleotides (6).

[6] Even in experiments run at 25°, when approximately 70 μmoles of ATP were synthesized oxidatively, there was still a dilution of the radioactivity of the intramitochondrial IP of approximately 20 per cent.

[7] It has been found that the phosphates of both phosphoproteins (24) and phospholipides (25) are very rapidly turned over in respiring mitochondria. We have found (unpublished observations) that, upon incubation of mitochondria in isotonic sucrose at 30°, all of the initial inorganic orthophosphate was lost from the mitochondria and no acid-insoluble phosphate fraction was lowered; when incubated with 0.01 M phosphate buffer (pH 7.2), approximately 20 per cent of the phospholipide phosphate and phosphoprotein phosphate was lost from the mitochondria (*cf.* (6));

Action of 2,4-Dinitrophenol—The specific activities obtained when various concentrations of DNP were added to the oxidative medium containing ADP are presented in Table V. As the concentration of DNP was increased, with increasing uncoupling of phosphorylation, the specific activities of both intra- and extramitochondrial ADP and ATP decreased (*cf.* (12, 21, 27, 28)). This effect of DNP also took place if AMP was substituted for ADP in the oxidative medium. The results would agree with the formulation of Hunter (29), of Lee and Eiler (30), and of Lardy and

TABLE V

Effect of DNP on Specific Activities and Quantities of Intra- and Extramitochondrial Nucleotides

Experimental conditions as for Table I, except that 30 μmoles of IP^{32} and approximately 60 μmoles of ADP were added. The theoretical IP uptake was 15.5 for zero DNP, 3.6 for 1×10^{-5} M DNP, and an IP discharge of 1.8 μmoles for 3×10^{-5} M DNP. The total intramitochondrial nucleotides were 5.47 μmoles for zero DNP, 5.40 μmoles for 1×10^{-5} M DNP, and 5.11 μmoles for 3×10^{-5} M DNP. The intramitochondrial nucleotide peaks (other than those represented in the table) remained at practically the same level with increasing DNP concentration, except the peak including TPN and IMP which increased 2-fold from zero DNP to 3×10^{-5} M DNP.

Concentration of DNP		IP		AMP		ADP		ATP		Ad-X	
		S.a.	μmoles	S.a.	μmoles	S.a.	μmoles	S.a.	μmoles	S.a.	μmoles
M											
0	Intramitochondrial	3510	3.6*	0	1.18	2170	0.96	3980	0.92	0	0.91
	Extramitochondrial	5950	9.3*	0	11.2	800	21.5	2230	26.6		
1×10^{-5}	Intramitochondrial	3130	4.8*	0	1.63	1430	0.70	1860	0.42	0	0.82
	Extramitochondrial	6650	20.3*	0	16.3	450	21.6	1100	19.9		
3×10^{-5}	Intramitochondrial	3160	4.7*	0	1.59	800	0.66	1050	0.37	0	0.72
	Extramitochondrial	5510	29.2*	0	18.8	130	22.8	220	17.0		

* Estimated through the radioactivities of all the tubes in the IP peak.

Wellman (28) in that little of the radioactive IP would be presumed to appear in ATP (under conditions of complete inhibition), but that an intermediate between IP and ATP (Y-PO_4 of Hunter (29)) would be broken down by DNP (see also (31–36)). The decrease in the specific activity of the intramitochondrial IP produced by DNP (also found by Abood (21)), small as it is, might reflect the discharge phenomenon of Teply (32), in that non-radioactive phosphate from some other source ("gel phosphate"

when incubated with DNP, the IP was lost completely, and approximately 40 per cent of the phospholipide phosphate and phosphoprotein phosphate was lost. Phospholipide and phosphoprotein phosphates were determined by the method of Schneider (26).

of Teply (32)) would dilute the added radioactive IP.[8] This effect would also be comparable to the "replacement" of added IP postulated by Loomis and Lipmann (33) and to the "sparing" of added IP postulated by Judah (34).

Site of Action of Added Hexokinase—In a previous paper (2), it was postulated that added yeast hexokinase acted in or on the mitochondria

TABLE VI

Effect of Exogenous Hexokinase on Specific Activities and Quantities of Intra- and Extramitochondrial Nucleotides and of Glucose-6-phosphate

Experimental conditions as for Table I, except that approximately 60 μmoles of ADP and 0, 20, and 40 μmoles of glucose were added to the appropriate flasks with the hexokinase solution. The G6P was isolated as described in "Experimental," and its specific activity is given as P^{32} c.p.m. per μmole of G6P determined by the anthrone test. The total ATP synthesized by oxidative phosphorylation could be calculated (assuming no breakdown of the G6P) to be 17.5 μmoles with zero hexokinase, 12.1 μmoles with 2 ml. of hexokinase, and 12.0 μmoles with 4 ml. of hexokinase.

Hexokinase added		IP		AMP		ADP		ATP		Ad-X		G6P	
		S.a.	μmoles	S.a.	μmoles	S.a.	μmoles	S.a.	μmoles	S.a.	μmoles	S.a.	μmoles
ml.													
0	Intramitochondrial	3200	5.3*	0	1.59	3480	0.94	6430	0.83	0	0.83		Trace
	Extramitochondrial	6640	48.7*	0	10.4	1460	18.2	3420	27.9				"
2	Intramitochondrial	3620	7.1*	0	1.60	3320	0.94	6160	0.78	0	0.87		"
	Extramitochondrial	5600	63.1*	0	17.0	1300	29.3	3070	12.1			2300	17.0*
4	Intramitochondrial	3640	7.7*	0	1.60	3340	0.80	5600	0.66	0	0.82		Trace
	Extramitochondrial	5630	63.2*	0	20.5	1250	35.3		2.4			2400	32.0*

* Estimated through the radioactivities of all the tubes in the IP peak.

in its stimulation of respiration. Since we have found conditions (Tables I, II, V) in which the intra- and extramitochondrial ATP can be differentiated, by the differences in specific activity, it was possible to delineate more closely the site of action of hexokinase. Table VI shows the result of an experiment in which G6P was collected as described in "Experi-

[8] It could be that DNP acts to prevent the initial "activation" of added IP by preventing its possible pooling with other sources of mitochondrial phosphate (see above), but the figures in Table V would seem to rule this out, since the radioactive dilution of intramitochondrial IP is still noted in the presence of DNP.

mental" and its specific activity compared with that of the intra- and extramitochondrial ADP and ATP. Since there is only a small breakdown of the G6P[9] once it is formed, its final measured specific activity is an integration of its initial specific activity of 0, from initially unlabeled ATP, to that of its maximal specific activity, from the radioactive ATP at the end of the experiment. Therefore, since the specific activity of the G6P fell between those of the high energy phosphates of the intra- and extramitochondrial ADP and ATP, it must mean that a preponderance of the phosphate of G6P must have come from the more highly radioactive intramitochondrial ATP. It can be seen from Table VI that, unlike the action of DNP (Table V), the presence of the hexokinase system produces only

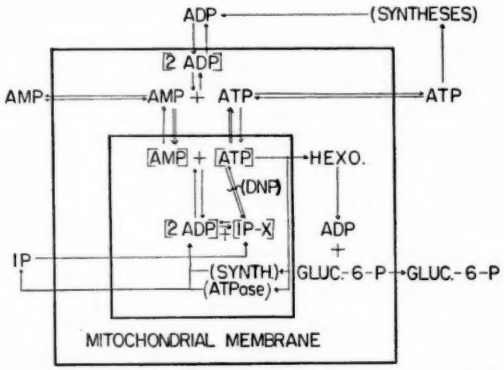


FIG. 2. Representation of biochemical structure of rat liver mitochondrion in terms of oxidative phosphorylation mechanisms.

small changes in the intramitochondrial nucleotides, including ATP. This indicates that the added hexokinase does not act on the intramitochondrial ATP (6, 40), but probably acts at the surface of the mitochondria, mostly on the ATP which has been formed within the mitochondria and is coming out into the medium (see Fig. 2). Ernster and Lindberg (41) have also postulated that the increased activity of hexokinase when mitochondria

[9] Even though the impure yeast hexokinase preparation had inorganic pyrophosphatase activity, there is far too little inorganic pyrophosphate in the flasks, or likely to be formed, to account for the observed inorganic phosphate output. Also, even though it has been found that the glucose-6-phosphatase of liver is associated with the microsome fraction (37), the presence of some activity in the mitochondria ((38), *cf.* (39)) could be significant in view of the larger amounts of mitochondria used. Thus, it was found that G6P added to the mitochondria could be split, and it could be calculated that in the centrifugation-incubation conditions employed approximately 2.5 μmoles of G6P would have been broken down.

are added to it might be due either to an activation of the enzyme by mitochondria or else that hexokinase acts on the mitochondrial surface, while Slater and Holton (42) found that free ATP formed from the endogenous ADP of heart muscle sarcosomes is capable of reacting with soluble proteins like hexokinase.

To test the possibility that yeast hexokinase added to a mitochondrial suspension might in part come down with the mitochondria upon centrifugation, experiments were performed in which hexokinase was mixed with mitochondria, and, after immediate centrifugation, the activity of the added hexokinase was compared with the activity in the mitochondria and medium fractions.[10] Only 90 per cent of the added hexokinase was recoverable in the medium, the other 10 per cent coming down with the mitochondria. Similar results showing absorption of added acid phosphatase by mitochondria, microsomes, and nuclei have been found by Berthet *et al.* (38). However, this 10 per cent of the added yeast hexokinase activity associated with the centrifuged mitochondria had from 3 to 9 times (according to the amount of hexokinase added) the activity of the added hexokinase (*cf.* (41)). It was found that the increased activity of this mitochondrially bound hexokinase was associated with a marked production of IP, which was proportional to the amount of the hexokinase originally added to the mitochondrial suspension. Since under these conditions it has been found that the mitochondria have very little ATPase activity, this IP output may well have come from the breakdown of G6P.[9] The reason for the increased activity of the hexokinase bound to the mitochondria is not known, but it might be due either to the increase in local concentrations of the reactants or to a relief of possible enzyme inhibition by the products (reports on yeast hexokinase inhibition by G6P are contradictory (43, 44)). It was also noted that insulin added to the mitochondrial suspension along with yeast hexokinase had no effect on the observed binding of the enzyme by the mitochondria.

Effect of Fluoride on Specific Activities of Intra- and Extramitochondrial Nucleotides—Since fluoride has been found to inhibit mitochondrial adenylate kinase (3–5), the action of F on the specific activity of the various nucleotides was tested. In the presence of IP^{32} and with either AMP or ADP as the initial nucleotide, no effect of 0.01 M F was noted on either the amount of oxidative phosphorylation observed or on the specific activity of the intra- and extramitochondrial nucleotides. It is not clear therefore that F acts in inhibiting oxidative phosphorylation under certain conditions (3, 6) solely by its observed inhibition of adenylate kinase.[11] The observed

[10] In contrast to the results of Crane and Sols (43), we have found very little liver hexokinase activity associated with the mitochondrial fraction.

[11] In extension of experiments mentioned earlier (5), we have found that the inhibition of adenylate kinase by 0.01 M F with much smaller amounts of mitochondria

lack of phosphorylation with AMP when the adenylate kinase of rat liver mitochondria was inhibited by F (3) or when rat heart muscle suspensions with low adenylate kinase activity were used (42), and the lag period of phosphorylation observed when AMP is added to a system with active adenylate kinase (4, 42), could be due to a necessity of activating AMP for entry into the site of mitochondrial phosphorylation. This might mean that, when ADP is added, the adenylate kinase at the mitochondrial surface (see Fig. 2) produces not AMP, but an AMP derivative, and the necessity for adding equimolar amounts of F to bind the derivative could explain the observed variations in the inhibitory effects of F.

DISCUSSION

Deductions regarding Structure of Rat Liver Mitochondria—From the results of Paper I (6) and of this paper, an attempt has been made to devise a representation of the biochemical structure of the mitochondria in terms of their oxidative phosphorylation mechanisms (Fig. 2). This diagram is based on the assumption that the two enzyme activities (adenylate kinase and oxidative phosphorylation) which come down together in the centrifuge are indeed present in the same mitochondrion. The intramitochondrial nucleotides are represented as being bound (as [ATP]) to some site within the mitochondrion (6). The mitochondria are represented as having an inner and an outer zone which could be interpreted to conform with the electron micrographs by Palade (45). The two sites of adenylate kinase postulated are shown on the diagram as being in the mitochondrial membrane or outer zone and also within the mitochondria at the site of the oxidative phosphorylation mechanism. It should again be mentioned that DNP is presumed to act between the hypothetical phosphate intermediate and [ATP] at the site of oxidative phosphorylation (Table V), that any ATPase activity of the mitochondria presumably takes place not at the surface of the mitochondria but within the mitochondria (Table III), as distinct from that of the ATPase of the yeast cell (46), and that added hexokinase acts mostly on or in the surface of the mitochondria on the ATP which was produced within the mitochondria and which is moving out into the medium (Table VI).

The data clearly show that during mitochondrial oxidative phosphorylation the turnover of phosphate in the intramitochondrial adenine nucleo-

(25 equivalent mg. of Mt) varies from 10 to 100 per cent in different experiments. Furthermore, in those oxidative experiments in which F lowered the P:O ratio when AMP was the initial nucleotide (3), there was no inhibition of the simultaneously measured adenylate kinase reaction, in either direction. In those experiments in which F did inhibit adenylate kinase, the inhibition was shown to be non-competitive in nature. In this connection the preincubation effect of F should also be noted (6).

tides under the conditions of the experiments mentioned is more rapid than the diffusion of the nucleotides in and out of the mitochondria. This conclusion is in agreement with that reached in an earlier paper (2) under different experimental conditions. Since it has been found ((6), see above) that the concentration of each of the adenine nucleotides within the mitochondria remained constant during active oxidative phosphorylation, it is possible that the rate of oxidative phosphorylation is regulated not only by the intramitochondrial (2), or extramitochondrial (2, 35, 40, 47, 48), turnover of the adenine nucleotides, but also by the rate of entry of phosphate acceptor, in the form of some adenine nucleotide, into the mitochondria. This regulation of the rate of entry might well be the function of the surface adenylate kinase (Fig. 2). The equilibrium constant of the adenylate kinase reaction can be calculated to be close to 1 (5), and, therefore, this enzyme could function effectively in the capacity suggested, since any small changes in concentrations of any of the reactants would shift the reaction very quickly. Reactions which tend to use ATP and produce ADP would, as a result of adenylate kinase action, make AMP or its product available for entry into the oxidative phosphorylation site. On the other hand, if ATP piled up, the adenylate kinase reaction would be shifted towards the formation of ADP; since this would reduce the amount of AMP present, it would reduce nucleotide entry and phosphorylation. This rate of entry of AMP into the mitochondria could well be the immediate means of the regulation of mitochondrial oxidation. Somewhat the same view has been expressed by Lindberg and Ernster (49), in that they postulated that AMP was indispensable for the maintenance of the generation of phosphate bond energy.

There is considerable evidence (see the review by Schneider (19)) that mammalian mitochondria contain a semipermeable, perhaps double (44, 45), membrane. It is conceivable that the enzyme adenylate kinase is a protein comprising part of this membrane. If we make the assumption that the membrane has little permeability to the adenine nucleotides, then this vantage point for adenylate kinase would confer a more specific and intimate means of regulating the oxidative rate of mitochondria.

Bartley and Davies (16) reported that mitochondria can accumulate Na^+, K^+, and various substrates, while these authors (16) and we[6] have found that the mitochondria can increase their concentration of IP. However, in the presence of AMP or ADP, non-oxidizing mitochondria can bring in, but cannot accumulate, these nucleotides, and oxidizing mitochondria cannot retain the ATP which they have synthesized from these nucleotides. In other words, the mitochondria act like a secretory body for the production of ATP. They can take the ATP from the intramitochondrial site of formation and use it at other sites within the mitochondria

for synthetic work (2) and for the transport of solutes across their membranes (50), or they can pass the ATP out to the rest of the cell for synthetic use there. It may be noted that recent reports by Harman and coworkers (51, 52) question the occurrence of mitochondrial membranes in the morphological sense. If their views are substantiated and extended to the mitochondrial material studied here, it will be desirable to consider what type of mitochondrial structure could yield the behavior found in the biochemical studies. The idea of inner zones and outer zones in the mitochondrial substance, which has been arrived at by purely biochemical studies, may prove to be an acceptable picture to both schools of morphologists. This view of mitochondrial structure appears to receive support from the recent studies of Glimstedt *et al.* who refer to matrix substance (53) and a granulated inner body (54) in rat liver mitochondria, in addition to an outer membrane.

SUMMARY

1. Mitochondria which had been carrying out oxidative phosphorylation in the presence of radioactive inorganic phosphate were separated from the incubation medium, and the specific activities of the intra- and extramitochondrial adenine nucleotides were determined.

2. It has been suggested from the results of these and similar experiments that there may be two sites of mitochondrial adenylate kinase activity, one at the surface of the mitochondria and the other at the site of oxidative phosphorylation within the mitochondria.

3. The results would indicate that during oxidative phosphorylation the turnover of the phosphates of mitochondrial ADP and ATP was greater than the passage of these compounds in and out of the mitochondria.

4. The data indicated that added inorganic phosphate mixes in the mitochondria not only with inorganic phosphate but with some other source of phosphate, and this mixture constitutes a pool of phosphate which is drawn upon during oxidative phosphorylation.

5. The results indicate that dinitrophenol acts on some phosphate intermediate between inorganic phosphate and adenosine triphosphate.

6. Added yeast hexokinase has been shown to act in part at the surface of the mitochondria upon the adenosine triphosphate which has been formed within the mitochondria and which is being released into the medium.

7. A scheme of the biochemical structure of the mitochondria as regards oxidative phosphorylation is presented, and the rôle of adenylate kinase is discussed in relation to the regulation of mitochondrial oxidation.

The authors are grateful for the help of Dr. R. B. Hurlbert, Dr. H.

XUM

Busch, Dr. H. Schmitz, and Miss A. Brumm in the technique of the ion exchange columns, and to Dr. B. C. Soyenkoff, New York University, for supplying us with the special reagent used in his phosphate method (8).

BIBLIOGRAPHY

1. Siekevitz, P., *Federation Proc.*, **13,** 297 (1954).
2. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **201,** 1 (1953).
3. Barkulis, S. S., and Lehninger, A. L., *J. Biol. Chem.*, **190,** 339 (1951).
4. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191,** 485 (1951).
5. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **200,** 187 (1953).
6. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **215,** 221 (1955).
7. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209,** 23 (1954).
8. Soyenkoff, B. C., *J. Biol. Chem.*, **198,** 221 (1952).
9. Koehler, L. H., *Anal. Chem.*, **24,** 1576 (1952).
10. Khym, J. X., and Cohn, W. E., *J. Am. Chem. Soc.*, **75,** 1153 (1953).
11. Berger, L., Slein, M., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29,** 379 (1946).
12. Green, D. E., Atchley, W. A., Nordmann, J., and Teply, L. J., *Arch. Biochem.*, **24,** 359 (1949).
13. Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V., and Hems, R., *Biochem. J.*, **54,** 107 (1953).
14. Cohn, M., *J. Biol. Chem.*, **201,** 735 (1953).
15. Boyer, P. D., Harrison, W. H., Falcone, A. B., and Gander, J. G., *Federation Proc.*, **13,** 185 (1954).
16. Bartley, W., and Davies, R. E., *Biochem. J.*, **57,** 37 (1954).
17. Novikoff, A. B., Podber, E., Ryan, J., and Noe, E., *J. Histochem. and Cytochem.*, **1,** 27 (1953).
18. Laird, A. K., Nygaard, O., and Barton, A. D., *Exp. Cell Res.*, **5,** 147 (1953).
19. Schneider, W. C., *J. Histochem. and Cytochem.*, **1,** 212 (1953).
20. Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201,** 245 (1953).
21. Abood, L. G., *Am. J. Physiol.*, **176,** 247 (1954).
22. Johnson, R. M., and Albert, S., *J. Biol. Chem.*, **200,** 335 (1953).
23. Griffiths, M., and Pace, N., *Proc. Soc. Exp. Biol. and Med.*, **83,** 771 (1953).
24. Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, **177,** 775 (1949).
25. Kennedy, E. P., *J. Biol. Chem.*, **201,** 399 (1953).
26. Schneider, W. C., *J. Biol. Chem.*, **161,** 293 (1945).
27. Sacks, J., and Sinex, F. M., *Arch. Biochem. and Biophys.*, **39,** 205 (1952).
28. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **201,** 357 (1953).
29. Hunter, F. E., Jr., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 297 (1951).
30. Lee, K.-H., and Eiler, J. J., *J. Biol. Chem.*, **203,** 705, 719 (1953).
31. Slater, E. C., *Nature*, **172,** 975 (1953).
32. Teply, L. J., *Arch. Biochem.*, **24,** 383 (1949).
33. Loomis, W. F., and Lipmann, F., *J. Biol. Chem.*, **173,** 807 (1948).
34. Judah, J. D., *Biochem. J.*, **49,** 271 (1951).
35. Potter, V. R., and Recknagel, R. O., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 377 (1951).
36. Potter, V. R., Siekevitz, P., and Simonson, H. C., *J. Biol. Chem.*, **205,** 893 (1953).

37. Hers, H. G., Berthet, J., Berthet, L., and de Duve, C., *Bull. Soc. chim.*, **33,** 21 (1951).
38. Berthet, J., Berthet, L., Appelmans, F., and de Duve, C., *Biochem. J.*, **50,** 182 (1951–52).
39. Kuff, E. L., and Schneider, W. C., *J. Biol. Chem.*, **206,** 677 (1954).
40. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195,** 215 (1952).
41. Ernster, L., and Lindberg, O., Résumé des communications, 2e Congrès International de Biochimie, Paris, 33 (1952).
42. Slater, E. C., and Holton, F. A., *Biochem. J.*, **55,** 530 (1953).
43. Crane, R. K., and Sols, A., *J. Biol. Chem.*, **203,** 273 (1953).
44. Wajzer, J., *Compt. rend. Acad.*, **236,** 2116 (1953).
45. Palade, G. E., *J. Histochem. and Cytochem.*, **1,** 188 (1953).
46. Rothstein, A., and Meier, R. C., *J. Cell. and Comp. Physiol.*, **32,** 77 (1948).
47. Niemeyer, H., Crane, R. K., Kennedy, E. P., and Lipmann, F., *Federation Proc.*, **10,** 229 (1951).
48. Rabinovitz, M., Stalberg, M. P., and Boyer, P. D., *Science*, **114,** 641 (1951).
49. Lindberg, O., and Ernster, L., *Exp. Cell Res.*, **3,** 209 (1952).
50. Bartley, W., Davies, R. E., and Krebs, H. A., *Proc. Roy. Soc. London, Series B*, **142,** 187 (1954).
51. Harman, J. W., and Kitiyakara, A., *Exp. Cell Res.*, in press.
52. Weinreb, S., and Harman, J. W., *J. Exp. Phys.*, in press.
53. Glimstedt, G., Lagerstedt, S., and Ludwig, K. S., *Exp. Cell Res.*, **7,** 575 (1954).
54. Glimstedt, G., Lagerstedt, S., and Ludwig, K. S., *Experientia*, **10,** 462 (1954).

THE

(F

Rec
drate
phate
enzyn
(2).
when
which
ulose
has b
by ex
elevat
An
the k
of rib
rats i

Ma
D-Xy
Treib
Glatt
Me
cal de
Uri
IR-12
umn
cent
below
Do
linke
sugar

* S
ships,

XUM

THE IDENTIFICATION OF RIBULOSE AND L-XYLULOSE IN HUMAN AND RAT URINE*

By SIDNEY FUTTERMAN and JOSEPH H. ROE

(From the Department of Biochemistry, School of Medicine, George Washington University, Washington, D. C.)

(Received for publication, December 27, 1954)

Recent work has indicated the importance of ketopentoses in carbohydrate metabolism. Horecker *et al.* (1) established that ribulose-5-phosphate is in equilibrium with ribose-5-phosphate in the presence of yeast enzymes. Phosphate esters of ribulose are formed during photosynthesis (2). Touster and associates (3, 4) have shown that glucuronolactone, when fed, is converted, both in man and the guinea pig, to L-xylulose, which is detectable in the urine. These authors also observed that L-xylulose was present in the urine of one of four normal human subjects. It has been shown that the urinary pentose excretion by the rat is increased by exposure to low temperature and thyroid feeding and decreased by elevated environmental temperature and thiouracil administration (5, 6).

An isolation procedure was devised which made it possible to identify the ketopentoses in rat and human urine. In this paper the occurrence of ribulose and L-xylulose in normal human urine and the urine of fasted rats is reported.

EXPERIMENTAL

Materials—Arabinose, ribose, and xylose were commercial preparations. D-Xylulose was prepared from D-xylose by the method of Schmidt and Treiber (7). D-Ribulose was prepared from D-arabinose by the method of Glatthaar and Reichstein (8).

Methods—Pentoses were estimated by the orcinol method (9), and optical densities were determined with a Beckman model B spectrophotometer.

Urine was desalted with ion exchange resins. A column of Amberlite IR-120, 48 cm. by 68 sq. cm., regenerated with 5 per cent HCl, and a column of Amberlite IR-4B, 38 cm. by 68 sq. cm., regenerated with 2 per cent NaOH, were both back-washed with water until the salt content was below 3 parts per million.

Dowex 1 anion exchange resin, 200 to 400 mesh and 8 per cent cross-linked, was converted to the borate form and used for the separation of sugars by the method of Khym and Zill (10). Paper chromatograms were

* Supported in part by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service.

XUM

developed by the ascending method of Williams and Kirby (11) and sprayed with aniline (12) or orcinol (13).

Isolation Procedure

880 ml. of pooled human urine, freshly collected from two normal subjects, were diluted to 2 liters and desalted by passage through columns of Amberlite IR-120 and IR-4B. The desalted urine was concentrated to about 5 ml., and aldoses were destroyed by oxidation to aldonic acids with Br_2 in the presence of excess $BaCO_3$ (14). Excess $BaCO_3$ was removed by centrifugation, excess Br_2 by aeration with CO_2, and the mixture was again desalted by passage through ion exchange resins. The effluent from the resin columns was concentrated to a syrup. The syrup was extracted three times with 5 ml. portions of ethanol, and a gummy residue which did not give a positive test with orcinol was discarded. The alcohol extract was concentrated to a syrup and dissolved in 10 ml. of a mixture consisting of 5 volumes of butanol, 5 volumes of acetone, and 2 volumes of H_2O. The solution was placed on a cellulose column, 20 cm. by 1.8 sq. cm., prepared from a slurry of cellulose in butanol-acetone-water, and the column was developed with butanol-acetone-water. Each 10 ml. fraction was tested with orcinol. The ketopentose fractions, Nos. 10 to 14, were pooled and concentrated to a syrup.

Separation of Ketopentoses—The syrup from Fractions 10 to 14 was dissolved in 10 ml. of 0.01 M $K_2B_4O_7$ and placed on a column of Dowex 1 resin in the borate form, 9 cm. by 1.57 sq. cm. Elution was started with 0.015 M $K_2B_4O_7$, and each 10 ml. fraction was tested with orcinol. Orcinol-positive Fractions 6 to 16 were pooled. After Fraction 25 was collected, elution was started with 0.05 M $K_2B_4O_7$ and orcinol-positive Fractions 30 to 34 were pooled. Each pooled fraction was concentrated to a syrup after removal of the $K_2B_4O_7$ (15).

With solutions of known sugars it had been observed that xylulose was eluted by 0.015 M $K_2B_4O_7$, while ribulose was retained on the column and could be eluted separately with 0.05 M $K_2B_4O_7$.

Identification of Ribulose—The syrup obtained from Fractions 30 to 34, eluted by 0.05 M $K_2B_4O_7$, was chromatographed on paper, and the results are presented in Table I. On each chromatogram the spot representing urine Fractions 30 to 34 migrated at the same rate as the standard ribulose spot.

The syrup was dissolved in water. The orcinol assay indicated a yield of 1.1 mg. of ribulose from urine Fractions 30 to 34, derived from the initial 880 ml. of urine. The orcinol spectra of the urine sugar and of synthetic ribulose (Fig. 1, Curves A and D) both showed two bands of approximately equal intensity, whose centers were at 540 mμ and 670 mμ respectively. Aldopentoses exhibit only the 670 mμ band.

Identification of L-*Xylulose*—The syrup obtained from Fractions 6 to 16, eluted from the Dowex 1 (borate) column with 0.015 M $K_2B_4O_7$, was chro-

TABLE I

Paper Chromatography of Ketopentose Fractions from Human Urine

The papers were developed for 16 to 20 hours. With the orcinol spray reagent (13) for ketoses, the standard xylulose spot and the spot given by Fractions 6 to 16 from the Dowex 1 (borate) column were both purple; the standard ribulose spot and the spot given by Fractions 30 to 34 from the Dowex 1 (borate) column were both pink.

Spot	Solvent system		
	Butanol 50 ml. + acetone 50 ml. + water 20 ml.		Phenol 90 gm. + water 10 gm.
	Orcinol spray (13)	Aniline spray (12)	Aniline spray (12)
	R_F	R_F	R_F
Arabinose		0.31	0.49
Xylose		0.35	0.45
Ribose		0.40	0.61
Ribulose	0.42	0.43	0.63
Urine Fractions 30–34	0.42	0.43	0.63
Xylulose	0.44	0.45	0.58
Urine Fractions 6–16	0.44	0.45	0.57

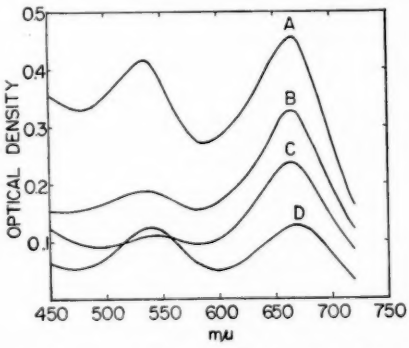


FIG. 1. Spectra of ketopentoses in the orcinol reaction (7). Curve A represents the spectrum obtained with urine Fractions 30 to 34, eluted from the Dowex 1 (borate) column with 0.05 M $K_2B_4O_7$. Curve B represents urine Fractions 6 to 16 eluted from the Dowex 1 (borate) column with 0.015 M $K_2B_4O_7$. Curve C was obtained with 12.8 γ of synthetic xylulose. Curve D was obtained with 7.4 γ of synthetic ribulose.

matographed on paper, and the results (Table I) indicated that the rate of migration of the spot representing urine Fractions 6 to 16 corresponded closely to that of synthetic xylulose.

The syrup was diluted to 2 ml. with water. The orcinol assay indicated a yield of 3.9 mg. of xylulose from Fractions 6 to 16. The orcinol spectra of the urine sugar and of synthetic xylulose (Fig. 1, Curves B and C) both show two absorption bands: a weak 540 mμ band and a strong 670 mμ band.

To the urine xylulose solution was added 0.4 ml. of a phenylhydrazine reagent, prepared by warming a mixture of 2 gm. of phenylhydrazine hydrochloride, 3 gm. of $CH_3COONa \cdot 3H_2O$, and 10 ml. of water. The mixture was heated in a boiling water bath for 45 minutes, and the osazone which formed was recrystallized from 30 per cent ethanol. The melting point of the urine osazone and the mixed melting points obtained with added D- and L-xylosazone are reported in Table II. The melting point of DL-xylosazone is about 40° higher than that of either enantiomorph.

TABLE II

Melting Points of Osazones

The osazone of the urine xylulose was prepared from the Dowex 1 (borate) column, Fractions 6 to 16, as described in the text.

Compound	M.p.
	°C.
Osazone of urine xylulose	162–164
D-Xylosazone	163–164
Mixed	199–201
DL-Xylosazone	203–206
Osazone of urine xylulose with added L-xylosazone	161–163

The rise in melting point observed when the urine osazone was mixed with D-xylosazone indicated that the urine sugar was L-xylulose.

Ketopentoses in Urine of Fasted Rats

Sixteen rats were fasted 24 hours, placed in clean cages, and urine was collected under toluene. Each morning the samples were pooled. The pH varied from 6.8 to 7.4 and was lowered to 6 by the addition of HCl. The urine was stored frozen. About 310 ml. of urine were collected in 4 days.

The rat urine was thawed, diluted to 2 liters, desalted by passage through columns of Amberlite ion exchange resins, and concentrated under reduced pressure. The residue was dissolved in 10 ml. of 0.01 M $K_2B_4O_7$ and the ketopentoses separated on the Dowex 1 (borate) column as described earlier. The syrup obtained from orcinol-positive Fractions 7 to 18, eluted by 0.015 M $K_2B_4O_7$, was examined by paper chromatography, and a spot corresponding to xylulose was observed. The syrup obtained from orcinol-

positive Fractions 30 to 38, eluted by 0.05 M $K_2B_4O_7$, was chromatographed on paper, and a spot corresponding to ribulose was detected.

DISCUSSION

The possibility was considered that the ketopentoses identified in the urine fractions might be produced by reactions occurring during the course of the isolation procedure. One such reaction might be the epimerization of aldoses to the corresponding ketoses. Accordingly, a mixture consisting of 1 gm. of urea, 1 gm. of NaCl, 10 mg. of ribose, 10 mg. of xylose, and 10 mg. of glucose was dissolved in water and taken through the isolation procedure. When the bromine oxidation step was omitted and the fractions from the Dowex 1 (borate) column were chromatographed on paper, only spots corresponding to the aldoses originally present in the mixture could be detected. Therefore, it seemed unlikely that the ketopentoses identified in the urine fractions were artifacts.

Since the ketopentoses were present in the urine of fasted rats as well as in normal human urine, it seemed improbable that they could be of dietary origin.

The possibility that the ketopentoses were synthesized by bacteria in the gut, absorbed, and excreted by the kidneys was not ruled out.

If the ketopentoses in urine are not of endogenous origin, it is conceivable, in the case of ribulose, that minute amounts of a phosphate ester might be hydrolyzed by an intracellular phosphatase. The free sugar might thus leak out of its metabolic pathway and be excreted by the kidneys. The detection of small amounts of L-xylulose in the urine of normal human subjects is in accord with the findings of Touster *et al.* (4) and supports the concept that pentosuria is an error of metabolism in which large amounts of a normal metabolite are excreted in the urine.

SUMMARY

Ribulose and L-xylulose have been identified in normal human urine. The yields in the isolation were approximately 1 mg. of ribulose and 4 mg. of xylulose per liter of urine. These sugars have also been detected in the urine of fasted rats.

Grateful appreciation is expressed to Dr. B. L. Horecker for valuable suggestions concerning this work.

BIBLIOGRAPHY

1. Horecker, B. L., Smyrniotis, P. Z., and Seegmiller, J. E., *J. Biol. Chem.*, **193**, 383 (1951).
2. Benson, A. A., Bassham, J. A., Calvin, M., Hall, A. G., Hirsch, H. E., Kawaguchi, S., Lynch, V., and Tolbert, N. E., *J. Biol. Chem.*, **196**, 703 (1952).

3. Touster, O., and Hutcheson, R. M., Abstracts, American Chemical Society, 126th meeting, New York, Sept., 84C (1954).
4. Touster, O., Hutcheson, R. M., and Reynolds, V. H., *J. Am. Chem. Soc.*, **76,** 5005 (1954).
5. Coover, M. O., Feinberg, L. J., and Roe, J. H., *Proc. Soc. Exp. Biol. and Med.*, **74,** 146 (1950).
6. Roe, J. H., and Coover, M. O., *Proc. Soc. Exp. Biol. and Med.*, **75,** 818 (1951).
7. Schmidt, O. T., and Treiber, R., *Ber. chem. Ges.*, **66,** 1765 (1933).
8. Glatthaar, C., and Reichstein, T., *Helv. chim. acta*, **18,** 80 (1935).
9. Albaum, H. G., and Umbreit, W. W., *J. Biol. Chem.*, **167,** 369 (1947).
10. Khym, J. X., and Zill, L. P., *J. Am. Chem. Soc.*, **74,** 2090 (1952).
11. Williams, R. J., and Kirby, H., *Science*, **107,** 481 (1948).
12. Partridge, S. M., *Nature*, **164,** 443 (1949).
13. Bevenue, A., and Williams, K. T., *Arch. Biochem. and Biophys.*, **34,** 225 (1951).
14. Seegmiller, J. E., and Horecker, B. L., *J. Biol. Chem.*, **192,** 175 (1951).
15. Zill, L. P., Khym, J. X., and Cheniae, G. M., *J. Am. Chem. Soc.*, **75,** 1339 (1953)

MICRODIFFUSION OF ACETIC ACID AS AN ASSAY FOR ACETYLCHOLINESTERASE*

By IRVING SERLIN and GEORGE C. COTZIAS

(From the Medical Department, Brookhaven National Laboratory, Upton, New York)

(Received for publication, December 4, 1954)

Acetic acid, one of the products of acetylcholinesterase (AChE) activity, may be measured directly within a wide range of concentration if it is first transferred into a second liquid of convenient volume. In such an arrangement, only the volume of the recipient water trap sets the level for the sensitivity of the procedure. In this paper a method is described which accomplishes this transfer by microdiffusion under conditions which protect the remaining acetylcholine (ACh) from non-enzymatic hydrolysis; a direct titration determines the amount of acetic acid. Determination of the activity by this direct analysis, rather than by difference from the amount of unchanged substrate (1), promotes procurement of accurate results, especially with small amounts of enzyme.

Rapid, quantitative diffusion of acetic acid from the reaction mixture is attained by adding the aqueous suspension to anhydrous sodium sulfate at low temperature. The sodium sulfate, under these conditions, rapidly forms a decahydrate and leaves the acetic acid free to diffuse into the second chamber of the microdiffusion unit.

Although the technique is presented here in its most convenient form, it should make possible the accurate determination of AChE activity under conditions which have hindered some previous work (2).

Method

Apparatus—(1) Porcelain Conway unit No. 1 with lid (Arthur H. Thomas Company); (2) tuberculin syringe, 1 ml. (Becton, Dickinson and Company), and No. 27 needle; (3) Rehberg micro burette (Alfred Bicknell Associates).

Reagents—(1) Potassium phosphate buffer,[1] pH 7.2, μ 0.1 (1.29 gm. of KH_2PO_4, 5.25 gm. of K_2HPO_4 per liter); (2) ampuls of acetylcholine chloride, 100 mg. (Merck and Company); (3) H_2SO_4, 0.0001 N; (4) citric acid monohydrate, 0.4 M (8.4 gm. per 100 ml.) (Mallinckrodt); (5) Na_2SO_4, anhydrous powder (Baker and Adamson); (6) $Ba(OH)_2 \cdot 8H_2O$, 0.005 N

* This work was supported by the Atomic Energy Commission.

[1] Tris(hydroxymethyl)aminomethane sulfate buffer, pH 7.2, 0.125 M, μ 0.19, can be substituted for phosphate buffer, provided that the molarity of the citric acid is changed to 0.15 M.

(789 mg. per liter of freshly boiled water); (7) phenol red in 5 per cent ethanol, saturated ($\approx$ 0.003 per cent).

Procedure—The contents of the ACh ampul are transferred into a 50 ml. volumetric flask and made to volume with the dilute H_2SO_4 to give 11 μmoles per ml. 1 ml. of a tissue homogenate in the phosphate buffer is placed in a 15 mm. $\times$ 120 mm. test-tube. After equilibration in a water bath at 37.5° $\pm$ 0.01°, 1 ml. of the ACh solution is added to the test-tube, the contents are mixed, and the time is noted. (A blank determination is run concomitantly, with the homogenate preheated in boiling water for 5 minutes.) At the end of 1 hour, the test-tube is immersed in ice-cold water and the reaction stopped by adding 0.2 ml. of the citric acid. The tube is transferred to a 4° cold room where a 1.0 ml. aliquot of the test (or blank) mixture is spread by means of the syringe on the outer ring of a Conway unit previously prepared as follows: The floor of the outer ring is covered with 4 to 7 gm. of Na_2SO_4 powder (stored at 4°), and 1.5 ml. of H_2O are placed in the central well. If some of the Na_2SO_4 powder spills into the water, or *vice versa*, no harm is done. After addition of the aliquot, the unit is sealed with a weighted, Vaseline-greased lid, brought to room temperature, and diffusion is allowed to proceed for 3 hours (less time for smaller samples). At the end of that time, 0.025 ml. (1 drop) of the indicator is added to the contents of the central well, and the latter is titrated with the $Ba(OH)_2$ to the end-point, which is a short lived appearance of a pink color.

The following arrangement has greatly facilitated this titration: The top of the Rehberg burette is joined through a 2-way stop-cock with a reservoir containing the $Ba(OH)_2$. The unit to be titrated is resealed with a special lid which has a 4 mm. hole drilled slightly off its center. The tip of the Rehberg burette as well as a fine capillary is admitted into the unit through that hole. The tip of the capillary is bent so that a stream of nitrogen is directed across the surface of the liquid in the central well. This arrangement provides for (*a*) stable $Ba(OH)_2$, (*b*) automatic mixing during titration, and (*c*) a sharp, durable end-point due to exclusion of the atmospheric CO_2.

1 unit of acetylcholinesterase activity is defined here as the quantity per ml. capable of catalyzing the release of acetic acid at the rate of 0.1 μmole per hour at 37.5°. If V_S and V_B are the volumes in ml. of 0.005 N $Ba(OH)_2$ required for the titration of the sample and blank, respectively, t is the time in hours, and V_A the volume in ml. of the aliquot tested, the number of enzyme units is given by $U = 55 \times (V_S - V_B)/t \times V_A$.

EXPERIMENTAL

The marked sensitivity of ACh towards acids made Conway's elegant acetic acid assay (3) inapplicable to the study of the acetylcholinesterases.

The use of citric acid to fix the pH at 3.0, in addition to stopping the enzymatic reaction and forming undissociated acetic acid, results in ACh stability, which is almost optimal. The acid hydrolysis of ACh is eliminated

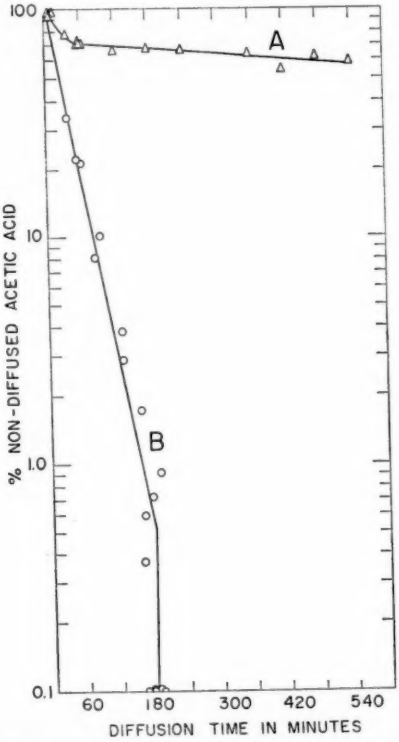


FIG. 1. Rates of diffusion of acetic acid from 1 ml. of test solution. Curve A, preparation of units and microdiffusions, both at 25°; Curve B, preparation of units at 4° and microdiffusions at 22–26°. The points on the abscissa represent recoveries of 100 to 101 per cent.

(as will be evident from the data on Fig. 2, Curve C), by the steps in the procedure which follow.

Previous workers (3, 4) have employed sodium sulfate to increase the vapor tension of acetic acid in aqueous solutions. A different approach seemed advantageous, however; namely, the use of the substantial drying capacity of anhydrous Na_2SO_4, 1 gm. of which, forming the decahydrate, would eliminate 1.27 ml. of water from the reaction mixture. This would act (*a*) to preserve ACh from acid hydrolysis, (*b*) to make for efficient de-

livery of acetic acid into the gas phase of the unit, and (*c*) to permit the use of larger aliquots for analysis.

The practicability of this approach was investigated with the behavior of acetic acid microdiffusion as a guide. An over-all, slow rate of transfer was found when 1 ml. aliquots of test solution were applied to anhydrous Na_2SO_4 at 25° (Fig. 1, Curve A). In most cases, recovery was incomplete, even after diffusion overnight. The very few units which gave quantitative recovery after 17 to 18 hours were invariably characterized by a completely dry appearance of the Na_2SO_4. Furthermore, the salt had set into a hard, solid form.

A search for more efficient drying agents, as judged again by their effect on the microdiffusion rate of acetic acid, led to investigation of the following compounds, singly or in combinations: $(NH_4)_2SO_4$, $CaSO_4$, $CdSO_4$, $CuSO_4$, $CoSO_4$, KNO_3, K_2SO_4, $MgSO_4$, $MnSO_4$, $MnSO_4 \cdot H_2O$, Na_2SO_4, $Na_2SO_4 \cdot 10H_2O$, Na_2SO_3, silica gel, borax, and oxalic acid. Anhydrous Na_2SO_4, however, performed best and seeding (5) with any of the other substances offered no advantages.

The conditions which promote rapid formation of $Na_2SO_4 \cdot 10H_2O$ were thereafter investigated more fully. Granules and powders of the anhydrous salt, varying in average particle size (down to the ones yielded by a 325 mesh sieve), were tested. The commercially available Baker and Adamson's anhydrous Na_2SO_4 powder (55 per cent of which passed through a 100 mesh sieve) was found markedly superior to all other forms tested and was used in this work.

The growth of hydrate crystals was observed under the microscope; these were seen to form most rapidly at temperatures considerably lower than that of the transition of sodium sulfate decahydrate to the anhydrous salt. The transition temperature (32.38°) can be markedly lowered by impurities (6), including excess of the ingredients of the enzymatic reaction mixture. Moreover, even with pure Na_2SO_4, the addition of water caused a marked elevation of temperature, approaching that of the transition. Accordingly, experiments were initiated at 4° with reagents and units equilibrated to that temperature; setting of the hydrate occurred within a minute. The diffusion (Fig. 1, Curve B) was subsequently carried out at 19–26° and went to completion within 3 hours, even in the presence of 25 mg. of tissue per ml.

Recovery of added acetic acid from solutions of pH 3.0 was also quantitative in the presence of 20 to 40 μmoles per ml. of the following *N*-acetyl compounds: acetylglycine (m.p. 205°), acetylalanine (m.p. 137–138°), *N*-2-hydroxyethylacetamide, and acetylanthranilic acid (m.p. 181–184°) (the latter partly in suspension). These compounds did not undergo hydrolysis

when handled in this manner. ACh chloride or sulfate,[2] in concentrations higher than 15 μmoles per ml., however, caused a delay in the diffusion of acetic acid. This created difficulties in experiments in which AChE was tested with high substrate concentrations. It became necessary, in those cases, to remove the ACh from the reaction mixture prior to microdiffusion analysis. This was accomplished by means of 200 mesh Amberlite IR-100 (H^+) resin. A column of this resin measuring 3 mm. × 55 mm. permitted quantitative separation of 1 μmole of acetic acid from 110 μmoles of ACh contained in 1 ml. of solution. A single wash with 1 ml. of water was

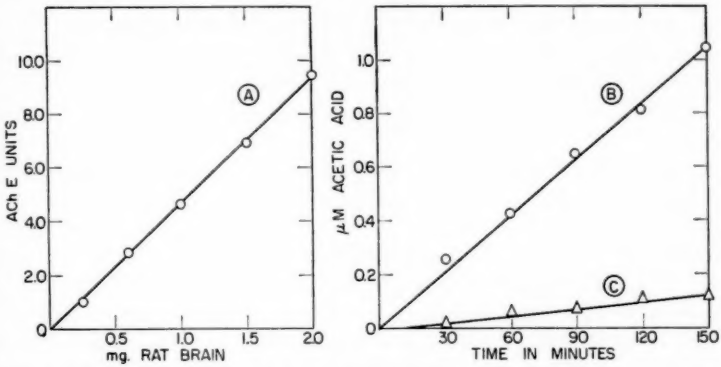


FIG. 2. Curve A, rate of acetic acid production from 5.5 μmoles of ACh as a function of the amount of enzyme. Curves B and C, relation of quantity of acetic acid produced from 5.5 μmoles of ACh to the time of incubation. Curves B and C demonstrate the rates of enzymatic and non-enzymatic hydrolysis of ACh, respectively. They are both proportional to the time of incubation and are not affected by the analytical procedure.

adequate for complete elution of the acid. In spite of the low pH of the eluate, hydrolysis of ACh, which was the rule with all other resins tested, did not occur with Amberlite IR-100 (H^+).

The microdiffusion procedure was applied to the assay of AChE and its competitive inhibition by physostigmine in rat brain homogenates (0.25 to 2 mg. per ml.), human and rat red blood cells (10 mg. per ml.), and a partially purified eel AChE preparation[3] (0.02 to 0.35 γ per ml.). Fig. 2 demonstrates parameters which make the enzyme the limiting factor to the acetic acid recovered.

[2] This salt was prepared from Merck ACh chloride solutions by passing them through columns of 40 to 50 mesh of Amberlite IRA-400 (sulfate form).

[3] Kindly supplied by Dr. David Nachmansohn.

The precision of the measurement was tested on twenty aliquots of a digestion mixture and its blank. The net enzymatic production of acetic acid was 0.0506 ± 0.0020 μmole per ml. per hour. The net non-enzymatic hydrolysis of ACh was 0.0290 ± 0.0011 μmole. The coefficient of variance was ±4 per cent for both sets of data. That this precision reflects also the accuracy of the method was shown by quantitative recoveries of added acetic acid: ten 1 ml. aliquots containing 0.9163 μmole of acid were analyzed with a mean and standard deviation of 0.9174 ± 0.0072 μmole.

The method, as presented here, was essential to a study of the inactivation kinetics of individual dilute AChE aliquots by irradiation.[4] The original activity of about 10 AChE units was made to decay to 0.5 unit; in spite of these 20-fold changes, the experimental data deviated from the constructed smooth curves invariably by less than 10 per cent.

SUMMARY

A simple, direct assay of acetylcholinesterase activity is presented. The procedure consists of a direct titration following microdiffusion of acetic acid from a reaction mixture, the pH of which has been fixed at 3.0.

Hydrolysis of the excess, labile substrate is prevented and diffusion of the acetic acid is expedited by addition of the sample to anhydrous sodium sulfate at 4°.

The procedure was found suitable for the assay of acetylcholinesterase preparations dilute enough to yield only 0.05 μmole (3 γ) of acetic acid per ml. per hour.

The microtitration set-up, which may be applied to all titrations in Conway units, was suggested and developed by James J. Greenough of this department.

BIBLIOGRAPHY

1. Hestrin, S., *J. Biol. Chem.*, **180,** 249 (1949). Martini, E., and Torda, K., *Klin. Wochschr.*, **16,** 824 (1937).
2. Augustinsson, K.-B., *Acta physiol. Scand.*, **15,** suppl. 52, 14 (1948). Nachmansohn, D., and Wilson, I. B., *Advances in Enzymol.*, **12,** 282 (1951).
3. Conway, E. J., Micro-diffusion analysis and volumetric error, London, 3rd edition, 246 (1950).
4. Bartley, W., *Biochem. J.*, **53,** 305 (1953).
5. Telkes, M., *Ind. and Eng. Chem.*, **44,** 1308 (1952).
6. Mellor, J. W., A comprehensive treatise on inorganic and theoretical chemistry, London, **2,** 667 (1922).

[4] Serlin, I., and Cotzias, G. C., unpublished results.

STUDY OF THE ENZYMATIC DEGRADATION OF DEOXYRIBONUCLEIC ACID BY TWO DIFFERENT DEOXYRIBONUCLEODEPOLYMERASES*

By M. PRIVAT DE GARILHE† AND M. LASKOWSKI

(From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin)

(Received for publication, December 6, 1954)

Pancreatic deoxyribonuclease (DNase I) is now available in crystalline form (2). The products obtained after a complete digestion of deoxyribonucleic acid (DNA) by DNase I have an average size of a tetranucleotide (3), but represent a complex mixture of many components, varying from mononucleotides (4–6) to oligonucleotides of an unknown size, estimated approximately as hepta- or octanucleotides (7). Sinsheimer (5, 7) isolated numerous dinucleotides and trinucleotides whose component nucleotides, but not their sequence, were identified. No definite statement as to the specificity of DNase I can be made at present.

A second enzyme (DNase II) capable of depolymerizing DNA, but different from DNase I, has been discovered by Maver and Greco (8) in spleen and thymus of calf. The existence of DNase II has since been confirmed by many workers (9–16), and methods for the partial purification of DNase II have been described (17–19). This enzyme differs from DNase I in respect to the pH optimum, the requirements for Mg^{++}, and the behavior toward the naturally occurring inhibitor for DNase I (20, 21). It was conceivable that the degradation of DNA by DNase II would lead to fragments different from those obtained with DNase I. The study of such fragments confirmed this expectation and is the subject of the present paper.

Methods

DNA was prepared by the method of Dounce *et al.* (22). Once crystallized, DNase I was either prepared by us by the method of Kunitz (2) or was purchased from the Worthington Biochemical Corporation. Partially purified DNase II was prepared according to the previously described method (19). Only the best batch of the potency of 2.0 has been used throughout this work. This preparation contained a trace of phosphodiesterase as measured by the method of Sinsheimer and Koerner (23),

* Supported by grants from the Atomic Energy Commission and the American Cancer Society. A preliminary report has been published (1).

† Fulbright Scholar. On leave of absence from the Sorbonne, Paris.

and easily detectable ribonuclease activity as measured by Kunitz' spectrophotometric method (24). The digestion of DNA was carried out in quantities of 200 to 500 mg. in a total volume of 5 ml. When DNase I was used, the solvent was 0.1 M borate buffer, pH 7.0, containing 0.025 M $MgSO_4$; when DNase II was used, the solvent was 0.1 M acetate buffer, pH 5.0. No criterion for the end-point of the reaction was considered satisfactory for the purpose of this work, since it was conceivable that, after the observable changes in viscosity had ceased, further degradation of the small fragments might proceed. It was arbitrarily decided to incubate 2 hours after the changes in viscosity stopped. In general, incubation lasted from 5 to 10 hours. Invariably a small precipitate of denatured protein appeared when the viscosity of the solution decreased. This precipitate was removed by centrifugation and was discarded, and the clear liquid was used for chromatography.

The chromatographic technique used for analysis of the digests was essentially that of Sinsheimer (7), with the exception that ammonium formate was used as the eluting agent instead of the original ammonium acetate. The formate buffer was prepared by titrating a solution of 10 M formic acid with ammonia to pH 4.5 and then diluting it to the indicated molarity.

The collected fractions were lyophilized and kept under a vacuum until all of the volatile buffer was removed. To facilitate this process, when dealing with large quantities of salt, the lyophilizing flasks were warmed to 40°. When the lyophilized fraction represented a pool of many tubes, on subsequent chromatography a fraction, brownish in color and strongly absorbing in the ultraviolet, appeared in the first few tubes. The ultraviolet spectrum of this fraction was different from any of the known derivatives of nucleic acids. The quantity of this substance was proportional to the number of tubes used for the pool and also to the concentration of the buffer in the pooled fraction. It was therefore assumed that this substance was eluted from the resin itself, and the first colored peak with an abnormal ultraviolet spectrum was always discarded from further consideration.

Results

The complete elution pattern of digest of DNA by DNase I is illustrated in Fig. 1. The initial part of the pattern which has been shown in the preliminary note (1) resembles the patterns published by Sinsheimer (7). In agreement with the statement of Sinsheimer (7), it was found that practically all of the digest (over 95 per cent) can be eluted with the 2 M buffer. It is interesting to note, however, that, of the peaks representing higher oligonucleotides, the largest quantitatively is that eluted with 1 M

buffer, while the peak eluted with 2 M buffer is significantly smaller. There are no oligonucleotides in the digest of DNase I, which, in order to be eluted, require a more concentrated buffer than 2 M.

Fig. 2 represents the results of an analogous experiment, with the exception that DNase II was used as an enzyme. The elution pattern is strikingly different from that shown in Fig. 1. Four mononucleotides are

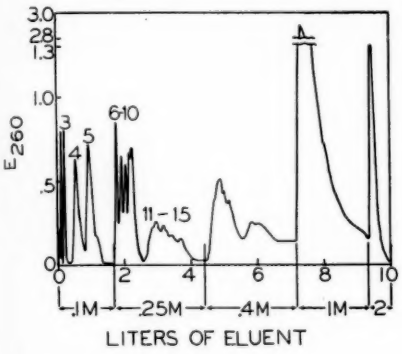


FIG. 1

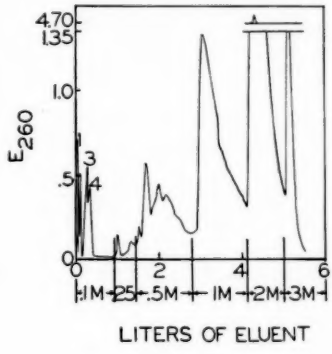


FIG. 2

FIG. 1. Elution pattern of the digest of 200 mg. of DNA with DNase I. Column, Dowex 1-2X (2 per cent cross-linked), 0.8 × 10 cm. Eluent, ammonium formate buffer, pH 4.5; volumes and molarity as indicated. The first two peaks eluted with 0.1 M buffer were Peak 1 (not labeled) cytidylic acid, Peak 2 (not labeled) thymidylic acid; Peaks 3, 4, and 5 were composed predominantly of dinucleotides of cytidylic acid; Peak 3, cytidylyl-cytidylic; Peak 4, cytidylic-thymidylic; Peak 5, cytidylic-adenylic. Peaks 6 to 10 (eluted with 0.25 M buffer) were mixtures of dinucleotides; Peaks 11 to 15 were mixtures of di- and trinucleotides.

FIG. 2. Elution pattern of the digest of 200 mg. of DNA with DNase II. Column, Dowex 1-2X, 0.8 × 10 cm. Eluent, ammonium formate buffer, pH 4.5; volumes and molarity as indicated. The first four peaks eluted with 0.1 M buffer were Peak 1, cytidylic acid; Peak 2 (not labeled) thymidylic acid; Peak 3, adenylic acid; Peak 4, guanylic acid.

present. There are practically no dinucleotides, very few trinucleotides, but considerably more of the higher oligonucleotides. The fraction eluted with 2 M buffer is much larger than an analogous fraction in the DNase I digest. The fraction eluted with 3 M buffer which was absent in the DNase I digest accounts for about 10 per cent of the material. This suggests that the major portion of the digest is composed of comparatively large oligonucleotides.

From a comparison of the two patterns (Figs. 1 and 2), it became obvious that very few of the fragments isolated from the two different digests were identical. It was then postulated that at least some of these frag-

ments may contain linkages susceptible to the action of a second enzyme. Two fractions from the DNase I digest, which were originally eluted with 1 M and 2 M buffers, were tried as substrates for DNase II. The digestion was carried out with a large amount of enzyme during 5 hours at 37°, pH 5, enzyme being added in 3 portions every 30 minutes until a total of 3 mg. was attained. Each of the digested fractions was then rechromatographed under conditions identical to the first chromatography.

The results of the second chromatography of these fractions are illustrated in Figs. 3 and 4. As previously stated (see "Methods"), an ultra-

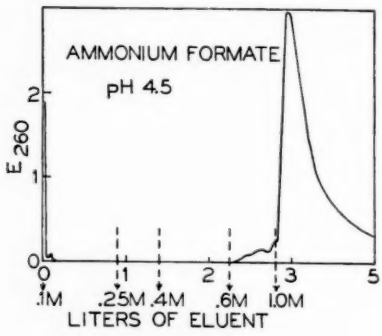


FIG. 3

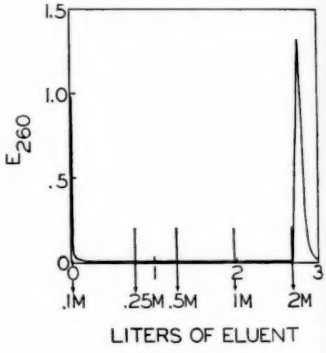


FIG. 4

FIG. 3. Chromatography of a fraction from a digest of DNA by DNase I, originally eluted with 1 M buffer, after the fraction had been subjected to the action of DNase II for 5 hours at 37°; pH 5.0. Chromatographic conditions as in Fig. 1.

FIG. 4. Chromatography of a fraction from a digest of DNA by DNase I, originally eluted with 2 M buffer, after the fraction had been subjected to the action of DNase II for 5 hours at 37°; pH 5.0. Chromatographic conditions as in Fig. 1.

violet-absorbing substance with a spectrum different from any of nucleic acid derivatives appeared in the first few tubes, but no mono-, di-, or trinucleotides appeared. Each of the fractions appeared at the same place in the chromatographic sequence as before the digestion, and the recovery for each of the fractions was over 90 per cent.

In order to ascertain that these fractions contain no linkages susceptible to either DNase II or DNase I, the fraction eluted with 1 M buffer was once again subjected to digestion with DNase II. This time small samples were withdrawn from the digestion mixture at hourly intervals and were placed on Whatman No. 3 filter paper. They were chromatographed with 70 per cent isopropanol-water-ammonia, according to the method of Markham and Smith (25). No evidence of digestion was found. The rest of the digest was then rechromatographed on the column under the condi-

tions identical with the previous experiment. The peak again emerged at the same place.

The recovered fraction was lyophilized and was subjected to digestion by DNase I. As previously, samples were withdrawn for paper chromatography and the rest of the digest was rechromatographed on the column. Both methods indicated that no digestion occurred.

The evidence was therefore conclusive that oligonucleotides from the digest of DNA by DNase I do not contain any linkages susceptible to the

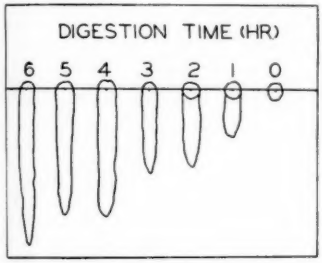


FIG. 5

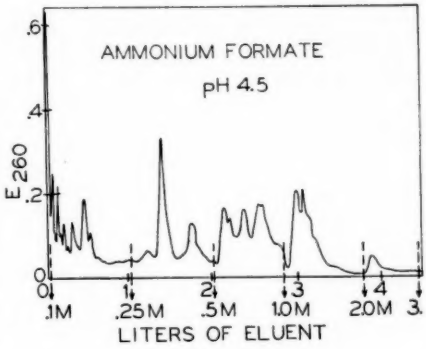


FIG. 6

FIG. 5. Progress of digestion followed by paper chromatography according to Markham and Smith (25). Aliquots taken at hourly intervals from the reaction mixture, in which a fraction from a digest of DNA by DNase II, originally eluted with 3 M buffer, served as substrate, and the enzyme was DNase I. The fraction was dissolved in 2 ml. of 0.1 M borate buffer, pH 7.0, containing 0.025 M $MgSO_4$; 0.5 mg. of DNase I in 0.1 ml. of buffer was added at hourly intervals.

FIG. 6. Elution pattern of the digest described in the legend for Fig. 5. Chromatographic conditions as in Fig. 1.

action of DNase II. Furthermore, these experiments indicated that the elution pattern illustrated in Fig. 1 represents the true end-products of the action of DNase I on DNA.

Experiments were then performed in which the fraction from DNase II digest, originally eluted with 3 M buffer and composed of higher oligonucleotides, was subjected to the action of DNase I. The digestion was followed by paper chromatography (Fig. 5), and the remainder of the digest was chromatographed on the column (Fig. 6). The pattern presented in Fig. 6 shows numerous di- and trinucleotides, and only a very small peak with 2 M buffer remains to represent higher oligonucleotides. Similar experiments performed with the fraction from DNase II digest, originally eluted with 2 M buffer, also indicated a significant degree of

hydrolysis. The results of these experiments lead to the conclusion that the large oligonucleotides obtained from the digest of DNase II are still good substrates for DNase I.

In an attempt to elucidate the presence of mononucleotides in the digest of DNase II, a series of experiments has been performed. Since our preparation of DNase II contained traces of phosphodiesterase as tested by the method of Sinsheimer and Koerner (23), it was conceivable that mononucleotides may have resulted from the action of this enzyme on dinucleotides.

Three dinucleotides, cytidylyl-cytidylic acid, a dinucleotide composed of adenylic and cytidylic acids, and a dinucleotide composed of thymidylic and cytidylic acids, were isolated from the digest of DNA by DNase I. Each dinucleotide was then subjected to the action of DNase II, first under the conditions favoring DNase II activity (pH 5.0; no Mg), then under the conditions favoring phosphodiesterase activity (pH 8.5; with Mg). All the results were negative; the dinucleotide always emerged at the same position and was not preceded by detectable amounts of mononucleotides.

Another experiment was performed as follows. DNA was digested with DNase I and the digest was chromatographed. Fractions originally eluted with 0.25 M buffer and representing di- and trinucleotides were pooled together, lyophilized once more, digested with DNase I, and rechromatographed. A pattern almost identical with the previous was obtained. The fractions were again pooled (with the exception of the first few tubes containing extraneous material from resin), lyophilized, digested with DNase II, and rechromatographed. The character of the pattern remained the same. No mononucleotide was observed.

DISCUSSION

If one postulates that DNase II degrades DNA into larger fragments than DNase I, the following results may be cited in support. A large part of the digest (over 40 per cent) remains on the column after elution with 1 M buffer. The fractions composed of larger oligonucleotides obtained after the DNase II digestion are good substrates for DNase I, but the analogous fractions obtained after DNase I digestion are resistant to DNase II. Both facts support the assumption that fewer linkages in DNA are susceptible to DNase II than to DNase I. They also imply that linkages susceptible to DNase II are usually widely separated in the intact DNA.

The only fact observed in this study not explainable on such an assumption is the presence of mononucleotides in the DNase II digest. The experiments in which di- and trinucleotides failed to give rise to mono-

nucleotides after incubation with the DNase II preparation suggest that mononucleotides arose from larger fragments by an unknown mechanism, whether the hydrolysis was catalyzed by DNase II or by a contaminant. Sinsheimer (26) reported that, with his best preparation of DNase II from spleen, no mononucleotides were found. This finding strongly favors the alternative that a contaminant is responsible for the appearance of mononucleotides. In such a case, the specificity of the contaminating enzyme may prove highly interesting.

SUMMARY

Beside the previously known differences between DNase I and DNase II, it was found that these two enzymes also differ in specificity. Oligonucleotides of higher molecular weight were found in the digest of DNA by DNase II than those found in the digest of DNA by DNase I. Di- and trinucleotides in the DNase II digest represented only a very small portion of the total digest, whereas they represented around 40 per cent of the total of the DNase I digest. The fractions composed of large oligonucleotides obtained after DNase II digestion were good substrates for DNase I but the reverse was not true. All these findings support the conclusion that fewer bonds in DNA are susceptible to the action of DNase II than to the action of DNase I. The appearance of mononucleotides in the digest after incubation with DNase II preparations was discussed.

BIBLIOGRAPHY

1. Privat de Garilhe, M., and Laskowski, M., *Biochim. et biophys. acta*, **14**, 147 (1954).
2. Kunitz, M., *J. Gen. Physiol.*, **33**, 349 (1950).
3. Kunitz, M., *J. Gen. Physiol.*, **33**, 363 (1950).
4. Sinsheimer, R. L., and Koerner, J. F., *J. Am. Chem. Soc.*, **74**, 283 (1952).
5. Sinsheimer, R. L., *Federation Proc.*, **11**, 287 (1952).
6. Potter, J. L., Brown, K. D., and Laskowski, M., *Biochim. et biophys. acta*, **9**, 150 (1952).
7. Sinsheimer, R. L., *J. Biol. Chem.*, **208**, 445 (1954).
8. Maver, M. E., and Greco, A. E., *J. Biol. Chem.*, **181**, 853, 861 (1949).
9. Webb, M., *Nature*, **169**, 417 (1952).
10. Brown, K. D., Jacobs, G., and Laskowski, M., *J. Biol. Chem.*, **194**, 445 (1952).
11. Alfrey, V., and Mirsky, A. E., *J. Gen. Physiol.*, **36**, 155 (1952).
12. Siebert, G., Lang, K., Lucius-Lang, S., Herkert, L., Stark, G., Rossmüller, G., and Jöckel, H., *Z. physiol. Chem.*, **295**, 229 (1953).
13. Cunningham, L., and Laskowski, M., *Biochim. et biophys. acta*, **11**, 590 (1953).
14. Kowlessar, O. D., Altman, K. I., and Hempelmann, L. H., *Arch. Biochem. and Biophys.*, **52**, 362 (1954).
15. Fellas, V. M., Meschan, I., Day, P. L., and Douglass, C. D., *Proc. Soc. Exp. Biol. and Med.*, **87**, 231 (1954).
16. Koszalka, T. R., Scheier, K., and Altman, K. I., *Biochim. et biophys. acta*, **15**, 194 (1954).

17. Webb, M., *Exp. Cell Res.*, **5**, 27 (1953).
18. Maver, M. E., and Greco, A. E., *Federation Proc.*, **13**, 261 (1954).
19. Laskowski, M., Steberl, E. A., Akka, R., and Watson, P., *Biochim. et biophys. acta*, **13**, 595 (1954).
20. Dabrowska, W., Cooper, E. J., and Laskowski, M., *J. Biol. Chem.*, **177**, 991 (1949).
21. Cooper, E. J., Trautmann, M. L., and Laskowski, M., *Proc. Soc. Exp. Biol. and Med.*, **73**, 219 (1950).
22. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., *J. Am. Chem. Soc.*, **74**, 1724 (1952).
23. Sinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198**, 293 (1952).
24. Kunitz, M., *J. Biol. Chem.*, **164**, 563 (1946).
25. Markham, R., and Smith, J. D., *Biochem. J.*, **52**, 558 (1952).
26. Sinsheimer, R. L., Abstracts, American Chemical Society, New York, 52C, Sept. (1954).

CORTICOTROPINS (ACTH)

VI. ISOELECTRIC POINT OF α-CORTICOTROPIN AS DETERMINED BY ZONE ELECTROPHORESIS ON STARCH*

By I. D. RAACKE and CHOH HAO LI

(From the Hormone Research Laboratory, University of California, Berkeley, California)

(Received for publication, November 6, 1954)

The peptide hormone (α-corticotropin), whose isolation from sheep pituitary glands has recently been described (2, 3), has been demonstrated to behave as a single chemical species on the basis of results obtained from stepwise degradation analyses of its *N*- and *C*-terminal sequences (4, 5) and from its amino acid composition (6), as well as from counter-current and chromatographic investigations (3). In this paper, the behavior of the peptide hormone when submitted to zone electrophoresis on starch will be reported.

EXPERIMENTAL

The electrophoretic apparatus was assembled with silver-silver chloride electrodes in appropriate buffer vessels (7), the details of which are to be described elsewhere. The procedures previously published for electrophoresis on starch have recently been reviewed by Kunkel (8). The starch was a commercial product (Swan), which was employed without purification; it was packed into small semicylindrical glass troughs (1.5 × 0.7 × 40 cm.), similar to those described earlier (9). Because of the size of the trough, only 0.05 to 1.0 mg. of the peptide hormone was required for each run. The starch in the trough was cut into 1 cm. segments, each of which was eluted with 2 ml. of distilled water. The protein content in the eluate was estimated by the method of Lowry *et al.* (10) with aliquots of 0.5 ml. All runs were performed with a potential difference of about 200 volts across the ends of the trough, as measured by a Simpson voltmeter, and were continued for approximately 24 hours in a cold room at 3°. Boundary electrophoretic experiments were carried out at 0° in a Perkin-Elmer model 38 of the Tiselius apparatus.

The buffers, of 0.1 ionic strength, containing 0.06 M NaCl, were all prepared from monovalent-monovalent salts. Specifically, they were as follows: acetate, pH 4.0 and 5.2; cacodylate, pH 6.6 and 7.0; and Veronal,

* Supported in part by grants from the National Institutes of Health, United States Public Health Service (G-2907), and from Eli Lilly and Company. For Paper V of this series, see Geschwind and Li (1).

pH 8.3. The α-corticotropin preparation was isolated from whole sheep pituitaries by a procedure previously described (2, 3). Crystalline preparations of ribonuclease, chymotrypsinogen, and bovine serum albumin were employed as reference proteins; ribonuclease and bovine serum albumin were generously supplied by Armour and Company, while the chymotrypsinogen, seven times crystallized, was kindly provided by Professor P. Desnuelle.

Electroosmotic Flow—For the determination of mobilities (u) by zone electrophoresis, it is necessary to know the displacement of the protein due to the electroosmotic flow (d_{el}). This may be accomplished by submitting two well known proteins to both boundary and zone electrophoresis and then calculating d_{el} from the expression

$$\frac{u_r}{u_s} = \frac{d_r - d_{el}}{d_s - d_{el}} \tag{1}$$

where u_r/u_s is the ratio of the mobilities, at the pH of the buffer in the starch trough, of two proteins, r and s, as determined by boundary electrophoresis, and d_r and d_s are the distances over which the proteins migrate on starch under identical conditions of time and field strength. Together with the data on the length of the trough (l in cm.), the voltage (V), and the time (t in seconds), u_{el} may be computed from the equation

$$u_{el} = \frac{d_{el}\, l}{Vt} \quad \text{or} \quad u_{el} = \frac{d_{el}}{Ft} \tag{2}$$

where F is the field strength (V/l). Table I summarizes the values of u_{el} obtained by investigations with two pairs of proteins; namely, ribonuclease-bovine serum albumin and chymotrypsinogen-bovine serum albumin. It is worthy of note that almost identical values were obtained by using different pairs of proteins.

Electrophoretic Mobility of α-Corticotropin—Fig. 1 gives the electrophoretic patterns (on starch) of α-corticotropin as investigated in buffers of different pH. In every case, the peptide migrated as a narrow zone, with no indication of the presence of contaminating components; furthermore, the ACTH activity coincided with the distribution of the peptide. This may be taken as supporting evidence for the homogeneity of the peptide hormone.

The electrophoretic mobility (u) was calculated from an expression similar to that of Equation 2,

$$u = \frac{(d_{obs} - d_{el})}{Ft} \tag{3}$$

where d_{obs} is the observed distance of migration and d_{el} is the displacement

due to electroosmotic flow as derived from the data in Table I by Equation 2; other symbols have their usual significance. This mobility may not represent the true mobility because of uncertainties, discussed later, involved in the determination of d_{el} and field strength in so complex a system. Table II presents the data employed for the calculation of the electrophoretic mobility of α-corticotropin in different buffers.

TABLE I

Electroosmotic Flow in Zone Electrophoresis on Starch Determined with Two Different Sets of Proteins

Buffer*	pH†	u_{el}‡ × 10^5, sq. cm. per volt per sec.	
		(*a*)	(*b*)
Acetate	4.9	1.20	
"	4.8		1.24
"	5.7	1.42	1.51
Cacodylate	6.8	1.39	
"	6.7		2.03
"	7.1	2.38	2.31
Veronal	8.1	2.23	2.22

* All buffers are of 0.1 ionic strength.

† pH as measured in the starch trough.

‡ The electroosmotic flow, u_{el}, is computed according to Equations 1 and 2, with two pairs of proteins. The values in Column (*a*) are obtained from the pair ribonuclease-bovine serum albumin, and those in Column (*b*) from the pair chymotrypsinogen-bovine serum albumin.

Isoelectric Point of α-Corticotropin and Other Proteins—In order to check the applicability of the present technique of zone electrophoresis to the determination of isoelectric points, three standard proteins (ribonuclease, bovine serum albumin, and chymotrypsinogen) were studied by both zone and boundary electrophoresis, and their isoelectric points in buffers of similar composition were determined by the two methods.

From the data obtained from free electrophoresis, the isoelectric points of ribonuclease, bovine serum albumin, and chymotrypsinogen in monovalent buffers of 0.1 ionic strength were found to be at pH 8.5, 4.6, and 8.1 respectively (see the broken lines in Fig. 2).

In order to obtain a cross-check on the correction term due to electroosmosis, the values of u_{el} used to correct the mobilities of chymotrypsino-

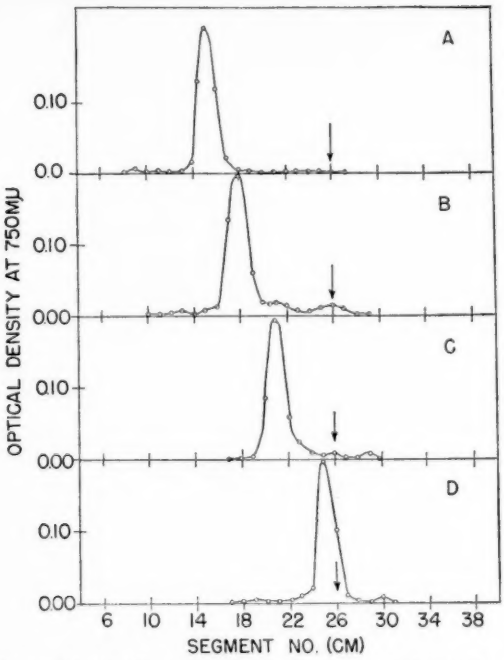


FIG. 1. Patterns obtained from zone electrophoresis on starch of α-corticotropin, in several different buffers, at the following pH values as measured in the trough: *A*, acetate, pH 4.8; *B*, acetate, pH 5.8; *C*, cacodylate, pH 7.2; *D*, Veronal, pH 7.8. The arrows indicate the site of application of the peptide hormone.

TABLE II

Electrophoretic Mobilities on Starch of α-Corticotropin

Buffer*	pH†	$F \times i \times 10^{-5}$	d_{el}	d_{obs}	u (10^5)
		volt × sec. per cm.	*cm.*	*cm.*	*sq. cm. per volt per sec.*
Acetate.....	4.85	3.59	4.3	10.6	1.75
"	5.80	3.59	5.1	8.3	0.89
Cacodylate.....	6.75	4.90	9.8	8.5	−0.26
"	7.10	3.69	8.7	5.5	−0.87
Veronal.....	7.80	3.60	8.1	0.5	−2.10

* All buffers of 0.1 ionic strength.

† pH as measured in the starch trough.

gen on starch were those which had been obtained from the pair ribonuclease-bovine serum albumin, whereas the mobilities of ribonuclease were corrected according to the results obtained from the pair chymotrypsinogen-bovine serum albumin. By plotting the corrected mobilities, calculated

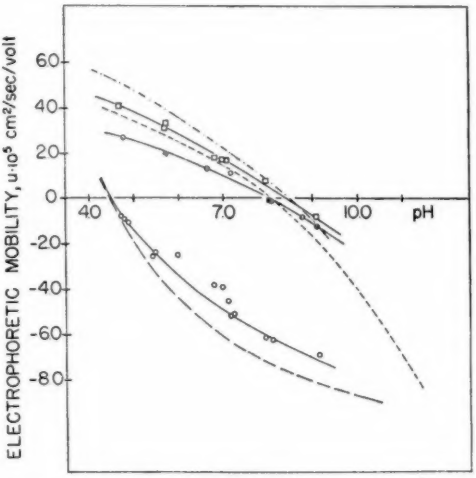


FIG. 2. Electrophoretic mobilities of three proteins as a function of pH. The solid lines refer to zone electrophoresis on starch, and the broken lines to boundary electrophoresis. □, ribonuclease; ⊙, chymotrypsinogen; ○, bovine serum albumin.

TABLE III

Comparison of Isoelectric Points Obtained from Starch and Moving Boundary Electrophoresis

Buffer	Ionic strength	Protein	Isoelectric points		Boundary	Value in literature
			(*a*)*	(*b*)†		
Acetate	0.01	Bovine serum albumin	4.6	4.15	4.55	4.59 (26)
						4.52 (11)
Veronal	0.10	Chymotrypsinogen	8.1	8.85	8.1	
"	0.01	"				9.5 (25)
Phosphate		Ribonuclease	7.7		7.8	7.8 (12)
Veronal	0.10	"	8.5		8.5	
"	0.01	"				9.45 (11, 25)

* Values obtained by the method described in the present investigation.

† Values obtained from comparison with the known isoelectric point of ribonuclease, according to Equation 4 (*cf.* (9, 14)).

according to Equation 3, against the pH of the buffer in the trough, the three proteins under investigation were found to give isoelectric points identical with those obtained from free electrophoresis (Fig. 2). As can be seen from Table III, these values are also in good agreement with those obtained by other investigators (11, 12). When the corrected mobilities of α-corticotropin of Table II are plotted against the pH of the buffers in the trough, an isoelectric point of 6.6 is obtained for the peptide hormone (Fig. 3).

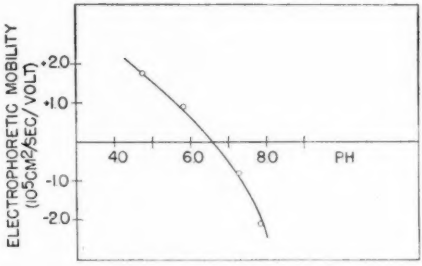


FIG. 3. Electrophoretic mobilities on starch of α-corticotropin as a function of pH.

DISCUSSION

The technique of zone electrophoresis, either on starch or on paper, which is so valuable for the investigation of peptides too small to be examined by free electrophoresis, or for the study of any substance available only in small amounts, has more or less serious limitations for obtaining quantitative data because of the necessity of applying corrections for the electroosmotic flow.

In paper electrophoresis, such corrections for the electroosmotic flow have generally been made by using as a point of reference a neutral substance, like dextran (13); this material, however, is not conveniently detected on starch and hence was not employed in the present investigation. Another method which has been used to correct for the displacement due to the streaming of buffer (9, 14) consists in running the unknown side by side with a protein of known isoelectric point (pI′) and calculating the isoelectric point of the unknown (pI) according to the formula

$$pI = pI_{obs} - (pI'_{obs} - pI') \quad (4)$$

where pI_{obs} and pI'_{obs} are the isoelectric points of the unknown and the standard protein, respectively, obtained by plotting the uncorrected mobilities or the distances of migration (provided the runs were made under identical conditions) *versus* pH. Such a procedure can give accurate results only if the pH-mobility curves for the two proteins are approximately

parallel and the electroosmotic flow corrections for the different buffers are of about the same order of magnitude. If these conditions are not satisfied, as they almost never are in practice, erroneous results will be obtained.[1] Further discussion of this procedure, as well as implications of the present method for the determination of mobilities, is to be presented elsewhere.

If a known protein is not run alongside the unknown, it is of great importance to employ a sufficiently standardized method so that predetermined correction factors can be used. The present method appears to satisfy the above conditions, since reproducible values, both for u_{el} and for mobilities in general, could be obtained under vastly different conditions.

It should be pointed out, however, that the corrected mobilities calculated according to Equation 3 by using the values of d_{el} obtained according to Equation 1 may not represent the actual mobilities, since they are always subject to the necessary condition that the ratio of the mobilities of the two proteins on starch be the same as the ratio of their mobilities in free solution. Since only the *ratio* of the mobilities has to be the same, the individual mobilities obtained by the two methods need not be the same, as indeed they are not, the mobilities on starch always being lower than those in free solution (see Fig. 2). However, the isoelectric points obtained by the two methods should be the same, as can be seen by the fact that, at the isoelectric point of one of the two proteins (say, r in Equation 1), the ratio u_r/u_s is equal to zero and hence d_r must equal d_{el}. This is found to be the case, as can be seen in Table III.

The recorded values for the isoelectric point of the pituitary adrenocorticotropic hormone (ACTH) have varied over the years according to the stage of purification of the particular preparation being studied. The earlier protein ACTH preparations (15, 16) had an isoelectric point of 4.7 as determined by moving boundary electrophoresis. Later, when it had become apparent that the active principle was peptidic in nature, the material containing the corticotropic activity was then investigated by means of paper electrophoresis. Using this method, Li (17, 18) found that the active ACTH principle was isoelectric in the neighborhood of pH 9 in borate buffers of 0.1 ionic strength. Unfortunately, the corrections for electroosmotic flow which were applied in these experiments were not adequate, and therefore the values obtained were high. Later determinations on paper with buffers of 0.015 ionic strength carried out by McDonald and Marbach (19) showed an isoelectric region between 6.0 and 6.6 for the active component. The spread of active material over a zone of considerable width could probably be attributed to the fact that very crude prep-

[1] As an example of this, note the isoelectric points of bovine serum albumin and chymotrypsinogen recorded in the fifth column of Table III, which were obtained according to Equation 4, with ribonuclease as the standard protein.

arations were used in these experiments. Similar observations were also made by Loomeijer and Witter (20) who found that the ACTH activity was carried along with the impurities and, consequently, was spread over the whole zone of migration.

Preparations of ACTH investigated by means of electrodialysis (21) and in exchange chromatography (22) appeared to be basic in nature. Moreover, the isoionic point of α-corticotropin, based on its amino acid composition and calculated according to the method of McLaren and Lewis (23), was also found to be basic; *i.e.*, 9.3 (6). It was therefore somewhat surprising to obtain an isoelectric point of 6.6 in the present investigation, and the finding merits consideration of the possible reasons for the discrepancy.

It is interesting to note that this experimental value of 6.6 coincides with a point of minimal solubility of the peptide (6). Furthermore, this discrepancy between the calculated isoionic point and the observed isoelectric point in buffers of relatively high ionic strength is by no means peculiar to α-corticotropin, but has been observed with many proteins. Just to cite a few of the most striking examples, when aldolase was investigated by Velick (24), its isoelectric point, determined in buffers of 0.1 ionic strength, were found to be in the neighborhood of 6, in contrast to a calculated isoionic point of 8.5 to 9.0. Similarly, both ribonuclease and chymotrypsinogen have a calculated isoelectric point in the neighborhood of pH 9.5, whereas the experimentally observed values for these proteins are considerably lower (see Table III).

The lowering of the isoelectric point of a protein with increasing ionic strength is generally attributed to binding of the buffer ions (24, 25). Hence, in a buffer of very low ionic strength, the experimentally determined isoelectric point should approach the theoretical one. In fact, Velick (24) reported that the isoelectric point of aldolase in buffers of 0.01 ionic strength is 8.5, while Anderson and Alberty (25) determined the isoelectric points of ribonuclease and chymotrypsinogen in such dilute buffers to be 9.5. The extrapolation of isoelectric points to zero ionic strength has not as yet been applied in the present investigation, partly because in zone electrophoresis on starch the electroosmotic flow becomes a very serious problem at lower ionic strength. It remains, therefore, to be determined whether the difference between the isoelectric point and the computed isoionic point of α-corticotropin is due to a large binding capacity on the part of the peptide hormone with respect to buffer ions or to some other peculiarity of the molecule.

SUMMARY

A procedure in which as little as 0.5 mg. of peptide or protein material is used for a single experiment has been described for the determination

of corrected electrophoretic mobility on starch. With crystalline ribonuclease, bovine serum albumin, and chymotrypsinogen as reference proteins, and the ratio of the mobilities, as determined by free electrophoresis, of any two of these compared with the ratio of the distances migrated on starch, the displacement due to electroosmotic flow was estimated. By this procedure, the isoelectric point of α-corticotropin in monovalent buffers of 0.1 ionic strength is found to be located at pH 6.6. The reasons for the discrepancy between this value obtained in buffered solution and the computed isoionic point (9.3) of the peptide hormone have been discussed.

BIBLIOGRAPHY

1. Geschwind, I. I., and Li, C. H., *Biochim. et biophys. acta*, **15,** 442 (1954).
2. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O., *Nature*, **173,** 251 (1954).
3. Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L., and Harris, J. I., *J. Biol. Chem.*, **213,** 171 (1955).
4. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **76,** 3507 (1954).
5. Harris, J. I., and Li, C. H., *J. Biol. Chem.*, **213,** 487 (1955).
6. Levy, A. L., Geschwind, I. I., and Li, C. H., *J. Biol. Chem.*, **213,** 187 (1955).
7. Raacke, I. D., and Li, C. H., *Biochim. et biophys. acta*, **14,** 290 (1954).
8. Kunkel, H. G., in Glick, D., Methods of biochemical analysis, Baltimore, **1,** 141 (1954).
9. Fønss-Bech, P., and Li, C. H., *J. Biol. Chem.*, **207,** 175 (1954).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
11. Alberty, R. A., Anderson, E. A., and Williams, J. W., *J. Phys. and Colloid Chem.*, **52,** 217 (1948).
12. Rothen, A., *J. Gen. Physiol.*, **24,** 203 (1940–41).
13. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, **35,** 89 (1951).
14. McDonald, H. J., Lappe, R. J., Marbach, E. P., Spitzer, R. H., and Urbin, M. C., *Clin. Chem.*, **5,** 35 (1953).
15. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **149,** 413 (1943).
16. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, **149,** 425 (1943).
17. Li, C. H., *J. Am. Chem. Soc.*, **74,** 2124 (1952).
18. Li, C. H., *Acta endocrinol.*, **10,** 255 (1952).
19. McDonald, H. J., and Marbach, E. P., *J. Biochem.*, Japan, **40,** 11 (1953).
20. Loomeijer, F. J., and Witter, A., *Acta endocrinol.*, **12,** 167 (1953).
21. Hess, G. P., Harris, J. I., Carpenter, F. H., and Li, C. H., *J. Am. Chem. Soc.*, **73,** 5918 (1951).
22. Dixon, H. B. F., Moore, S., Stack-Dunne, M. P., and Young, F. G., *Nature*, **168,** 1044 (1951).
23. McLaren, A. D., and Lewis, C., *J. Polymer Sc.*, **5,** 379 (1950).
24. Velick, S. F., *J. Phys. and Colloid Chem.*, **53,** 135 (1949).
25. Anderson, E. A., and Alberty, R. A., *J. Phys. and Colloid Chem.*, **52,** 1345 (1948).
26. Longsworth, L. G., and Jacobsen, C. F., *J. Phys. and Colloid Chem.*, **53,** 126 (1949).

XUM

TH

(*Fr*

In
they f
gestio
this q
colori
of the

The
nated
metho
cobalt
for co
metho
determ
to dev

Rea

1. S
scribe
100 m
water
ter pa

2. V

* Th
Medica
ciety, C

[1] If
tory, th
positio

[2] Th
ney tub

[3] In
sorptio

XUM

THE CONCENTRATION OF POTASSIUM IN GLOMERULAR URINE OF NECTURI*

By P. A. BOTT

(From the Department of Physiological Chemistry, Woman's Medical College of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, December 13, 1954)

In 1924 Wearn and Richards (2) reported that with qualitative tests they found potassium in glomerular urine of frogs, and it was at the suggestion of Dr. Richards, and in his laboratory, that the first beginnings of this quantitative work on potassium were made.[1] This paper describes a colorimetric ultramicromethod for potassium and its use in a comparison of the potassium concentrations of serum and glomerular fluid in *Necturi*.[2]

Analytical Method

The analysis consists of an ultramicro adaptation of the method originated by Kramer and Tisdall (5) and later modified by Hoffman (6), whose method involved (*a*) the precipitation of the potassium as sodium potassium cobaltinitrite, and (*b*) the use of a constituent of the dissolved precipitate for colorimetric estimation. The principal novelties in the ultramicromethod are the use of capillary apparatus, which may also be valuable for determining other bases, and the use of benzidine plus α-naphthylamine[3] to develop the color with nitrite in the last step.

Reagents—It is advisable to use redistilled water throughout.

1. Sodium cobaltinitrite solution. This formerly was prepared as described by Kramer and Tisdall (5) but now is prepared each day as follows. 100 mg. of reagent grade sodium cobaltinitrite are dissolved in 0.5 ml. of water in a small test-tube. The solution is filtered through a 40 mm. filter paper into another small test-tube and kept stoppered.

2. Wash Solution A. 10 per cent ethyl alcohol saturated with pure

* This investigation was supported in part by a grant from the Life Insurance Medical Research Fund. Reported in part before the American Physiological Society, Cleveland, April, 1951 (1).

[1] If a substantial part of this work had been completed in Dr. Richards' laboratory, this would have been Paper XVI in the series, "Quantitative studies of the composition of glomerular urine."

[2] The method has also been applied to samples of amphibian and mammalian kidney tubule fluid (3, 4).

[3] In our hands the use of this mixture resulted in a color with greater light absorption than any of those usually used in macro procedures.

XUM

$K_2NaCo(NO_2)_6$ as described by Hoffman (6). Only a few ml. need be filtered off for use each day.

3. 70 per cent ethyl alcohol.

4. 30 per cent acetic acid.

5. Saturated sodium acetate. Reagent grade sodium acetate in water solution.

6. 1 per cent benzidine dihydrochloride solution. This reagent is shaken at intervals for a few hours and filtered into a brown bottle. The solution keeps for at least a month.

7. 0.5 per cent α-naphthylamine in 30 per cent acetic acid. This solution is pale pink in color and should be discarded when the color becomes dark.

Procedure—The capillary tubing and method of measuring fluid columns under the microscope are as described by Richards and his associates (7, 8). Each micrometer scale division is equal to 0.1 mm. and a column of fluid 10 divisions long in the "small" constant diameter capillary tubing is equal to a volume of approximately 0.1 μl. In this method the procedure is identical for watery solutions and for serum.

A column of saturated sodium acetate solution 10 divisions in length is measured in a small capillary tube and drawn in 2 cm. from the end. This is followed by a column of the sample 20 divisions in length, which is drawn in 1 cm. from the end. When the tubing is removed from the microscope stage and cut off at about 6.5 cm., it appears as in Fig. 1, *A*, with the sample near the bottom. This tube is then lowered carefully into the hairpin turn or "U" device shown in *B*, made of slightly larger Pyrex tubing, except for the curved part, which is made of flexible plastic tubing.[4] The inner capillary tube is fastened to the outer by means of de Khotinsky cement so that the combined capillaries appear as in *C*, held in a small test-tube. By means of rubber-tipped forceps this assembly is lowered into a centrifuge cup. It is spun rapidly for 6 to 8 seconds to force and wash the sample into the bottom of the U. The larger tubing is cut off just below the cement seal, making it possible to lift out the inner tube. A second small capillary is used to measure a 3.5 mm. column of the cobaltinitrite reagent which is delivered into the U in the same way. The top of the outer capillary tube is rounded in a micro flame after the tube has been cut off again for removal of the inner capillary. The plastic

[4] This was No. 24 "transflex" tubing manufactured by the Irvington Varnish and Insulator Company, Irvington, New Jersey. Less precipitate is lost in removing supernatant fluid when the plastic tubing is used. This is so probably because the precipitate adheres to this tubing better than to glass, and because the glass shoulders help to hold back any precipitate which creeps along the curved plastic tubing.

tubing of *D* is fitted over this flamed end and, while the analyst holds the assembly in one hand, the sample and reagent are rocked back and forth in the bottom of the U by gentle interrupted pressure on the rubber tubing of *D* with the thumb and forefinger of the other hand. When the fluids are thoroughly mixed, the assembly is allowed to stand for an hour in a wooden block placed in a humidified cabinet.

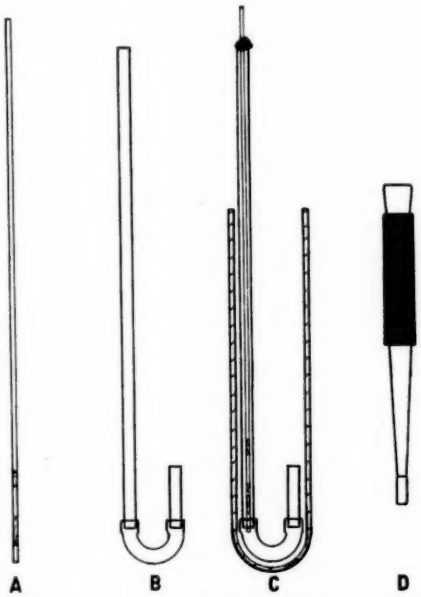


FIG. 1. Apparatus for ultramicroprecipitation and washing of sodium potassium cobaltinitrite. *A*, Pyrex capillary (outside diameter, 0.5 mm.) holding the sample and sodium acetate solution; *B*, capillary hairpin made of a 1.4 cm. length of plastic tubing attached to two pieces of Pyrex tubing 0.6 mm. in inside diameter, one of which is 6.0 and the other 0.7 cm. long. A small sturdy test-tube supports the assembly in *C*. *D*, piece of rubber tubing with a glass plug at the upper end and a glass adapter fitted with plastic tubing at the lower end.

After the period of standing, a 5.0 mm. column of water is measured and delivered into the U as in the case of the other reagents, the tube is cut off and flamed as before, and the whole assembly centrifuged for 5 minutes. While a folded piece of filter paper is being held against the open short arm of the U to absorb extruded liquid, the supernatant fluid is pushed very slowly out of this arm by pressure on the rubber tubing of device *D* attached as before. Under the microscope the short arm of the

U is filled with wash solution *A*, which is transferred from a micro pipette. After a 1 minute centrifugation, this wash fluid is expelled in the same way as was the supernatant reagent. The same procedure is then used for two washings of 70 per cent alcohol.

The capillary hairpin is removed from the test-tube and is held by the long arm, while the short arm and plastic part are rinsed with water on the outside and dried by touching with the edge of a piece of filter paper. The long glass arm is removed and discarded, and the short arm is cut off about 1 mm. from the plastic. The remainder, containing the precipitate, is dropped from a fine pair of forceps into a 10 × 75 mm. test-tube containing 0.8 ml. of water.

Although up to this point samples are handled singly, second and third samples may be started during the 1st hour period of standing. In this way the subsequent procedures can be carried out at the proper time for each sample. It is impossible to do this with more than three samples at a time because of the required timing. When the plastic tubings carrying the sodium potassium cobaltinitrite have been placed in water in separate test-tubes, they are handled as usual with a series of tubes. The tubes are spun for a few seconds in a centrifuge to remove any air which may be trapped inside of the plastic tubing and to insure a free flow of water through them. They are placed in a small beaker containing a half-inch layer of water and brought to boiling. Heat is removed, but the tubes are allowed to stand in the bath for a few minutes. The entire time for heating and standing is 10 minutes. The tubes are cooled in running water, centrifuged briefly again to remove air bubbles, and placed in a rack which also holds a "blank" tube containing 0.8 ml. of water.

The development of color is as follows: 0.1 ml. of the acetic acid is added to each tube from a fine tipped pipette. This is followed by 0.2 ml. of the benzidine dihydrochloride solution and, after 3 minutes, by 0.1 ml. of α-naphthylamine solution. The tubes are stoppered and allowed to stand for 25 minutes for the development of a lavender color. The colored solutions are then transferred to photometer tubes or cells,[5] and the absorption of light of wave-length 550 mμ is measured after setting the instrument with the reagent blank. Evaluation of these readings is made on curves plotted by taking the readings of standard potassium solutions run through the identical procedures described. The standards contained 50 m.eq. per liter of sodium in addition to from 0 to 10 m.eq. per liter of potassium. For a sample containing 10 m.eq. per liter (0.078 γ per 0.2 μl. of sample) of potassium, the optical density per cm. of light pathway is approximately 0.25.

[5] The micro cell of the Evelyn colorimeter and the small test-tubes of the Coleman junior spectrophotometer have both been used for this purpose.

Results

Since, in analyzing glomerular or tubule fluid, a single sample is often all that can be spared for analysis, the figures to be given for the control ultramicro analysis are those of single determinations. The comparison shown in Table I indicates the agreement of results obtained by the flame photometer method as described by Fox (9), and ultramicro analysis on two types of serum. Approximately 0.2 μl. was used for the ultramicromethod. The errors of recoveries of potassium added to two samples of serum were −3 and +4 per cent by the ultramicromethod. On six aqueous solutions, the potassium content of which was between 3 and 7 m.eq. per liter but was unknown to the analyst, the average error was 3.5 and the maximal error 6.5 per cent. In the comparison of concentrations of glomerular fluid and serum to be described, the *relative* error is probably less than that of these absolute values, since portions of the same piece of capillary tubing can be used for the three samples.

TABLE I

Comparison of Results Obtained on Serum with Flame Photometer and Ultramicromethods

Type of sample	Flame photometer*	Ultramicro	Difference
	m.eq. per l.	*m.eq. per l.*	*per cent*
Necturus serum	3.28	3.35	+2.1
" "	3.28	3.10	−5.5
" "	3.28	3.35	+2.1
" "	3.59	3.50	−2.5
" "	3.59	3.80	+5.8
" " + added potassium	6.15	5.90	−4.1
" " + " "	6.15	6.15	0.0
Rat serum	5.20	5.00	−3.9
" "	5.00	4.88	−2.4
" "	4.72	4.57	−3.2
" "	5.80	5.65	−2.6

* Sample size from 0.1 to 1 ml.

Animal Experiments

The technique used for collection of glomerular fluid was that of Wearn and Richards (2), and in general it was used on *Necturi* as described by Walker and Reisinger (10). Certain differences in the procedure were as follows. In most of the experiments capillary blood samples were taken from the postcaval or mesenteric vein shortly before the collection of glomerular fluid began and from the aorta after the collection was stopped.

It was felt that the potassium concentration of arterial blood serum should be compared with that of glomerular fluid, but some difficulty was encountered with circulation to the kidney when blood was taken from the aorta before collection. Since, as can be seen in Table II, the venous and arterial serum concentrations were so nearly identical, their averages were used. Results of the experiments would have been practically the same in most cases if the arterial samples only had been used. Heparin was used to prevent clotting of blood in the first three experiments, but was omitted in the others. The capillary blood tubes were centrifuged for 2 minutes immediately after the blood was drawn, and the portion of the

TABLE II

Potassium Concentration of Blood Serum (or Plasma) and Glomerular Fluid of Necturus

Experiment No.	Rate of fluid collection	Heparin used	Potassium concentration				Difference
			Serum I	Serum II	Average	Glomerular fluid	
	μl. per hr.		*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*	*per cent*
1	0.9	+	3.4	3.4	3.4	3.5	+2.9
2	0.4	+	4.0	4.2	4.1	4.1	0
3	0.5	+	3.1	3.3	3.2	3.3	+3.1
4	>0.5	−	3.5	3.2	3.35	3.3	−1.5
5		−	4.0	4.0	4.0	3.9	−2.5
6*	0.6	−	5.1	5.1	5.1	4.9	−3.9
7*	0.2	−		3.65		3.5	−4.1
8*	1.6	−	4.5	4.3	4.4	4.1	−6.8
Mean							−1.6

* The kidney was covered with a thin layer of paraffin oil.

tube containing the cells was cut off. In the last three experiments 0.5 ml. of 3.3 per cent mannitol in distilled water was injected beneath the loose skin under each fore leg of the animal before the incision was made.

In the eight experiments shown in Table II, the greatest difference between the potassium concentration of plasma and glomerular fluid was 6.8 per cent. In this experiment the difference would have been less if the arterial blood serum only had been used. Since the per cent difference depends on the analysis of three fluids, this difference seems well within the total error of the experiments. The potassium concentration of glomerular fluid in the kidney of *Necturus* appears, therefore, to be essentially the same as that of serum or plasma. In view of the low concentration of protein in the plasma of *Necturus* (11), this would be expected of an ultrafiltrate of plasma, provided that the potassium is in filtrable form.

SUMMARY

1. A colorimetric ultramicromethod for the determination of potassium has been described. The principle of the method is the precipitation of potassium as sodium potassium cobaltinitrite in a capillary "U" tube, and the photoelectric determination of nitrite in a solution of this salt by means of the color reaction with α-naphthylamine and benzidine. With samples 0.2 μl. in size, containing from 0.027 to 0.078 γ of potassium, the average error was approximately 3.6 per cent.

2. In seven out of eight experiments on *Necturi* the potassium content of glomerular fluid was within 4.1 per cent of that of serum or plasma. In one the difference was 6.8 per cent. The results have been interpreted as indicating that in the kidney of *Necturus* plasma potassium is freely filtrable through the glomerular membranes.

The author wishes to express her gratitude for the help and encouragement which Dr. A. N. Richards has given her in this work.

BIBLIOGRAPHY

1. Bott, P. A., *Federation Proc.*, **10**, 165 (1951).
2. Wearn, J. T., and Richards, A. N., *Am. J. Physiol.*, **71**, 209 (1924).
3. Bott, P. A., in Bradley, S. E., Renal function, Josiah Macy, Jr., Foundation, New York, 42, 5th conference (1954).
4. Wirz, H., and Bott, P. A., *Proc. Soc. Exp. Biol. and Med.*, **87**, 405 (1954).
5. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, **46**, 339 (1921).
6. Hoffman, W. S., *J. Biol. Chem.*, **120**, 57 (1937).
7. Richards, A. N., Bordley, J., 3rd, and Walker, A. M., *J. Biol. Chem.*, **101**, 179 (1933).
8. Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107**, 661 (1934).
9. Fox, C. L., Jr., *Anal. Chem.*, **23**, 137 (1951).
10. Walker, A. M., and Reisinger, J. A., *J. Biol. Chem.*, **101**, 223 (1933).
11. White, H. L., *Am. J. Physiol.*, **90**, 689 (1929).

STUDIES ON THE CONJUGATED LIPIDES

VI. ON THE STRUCTURE OF INOSITOL PHOSPHOLIPIDE*

By EIJI OKUHARA and TEISHI NAKAYAMA

(From the Department of Medical Chemistry, School of Medicine, Hokkaido University, Hokkaido, Japan)

(Received for publication, December 14, 1954)

It was first suggested by Anderson (1) that an inositol-containing phospholipide was present in the tubercle bacillus. Later a phospholipide of this type was isolated by Klenk and Sakai (2), Woolley (3), and Folch and Woolley (4) from the ordinary phospholipide fractions of soy bean and brain respectively. Further studies have been carried on by many workers on this lipide and its components in plant and animal tissues.

According to Carter and Celmer (5), inositol-containing lipides so far isolated from plant sources are mixtures containing carbohydrate and nitrogenous components which are difficult to separate. Moreover, Folch (6, 7) reported that inositol-containing lipides of soy bean and brain differ in that the former on hydrolysis yields inositol monophosphate and the latter inositol diphosphate. However, Folch was mainly concerned with the inositolphosphoric acid ester, leaving the elucidation of the chemical structure of this phospholipide to further investigation.

In this laboratory Imai (8) has been engaged in the study of inositol-containing lipide in soy bean for several years. We have extended his work and have tried to obtain the inositol phospholipide in purer form. We soon found that the nitrogen content of this phospholipide was almost negligible, suggesting that it does not contain a nitrogenous base.

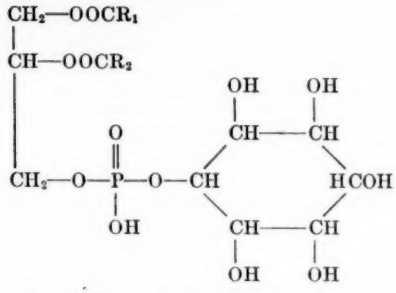
From this and from the findings of Folch (6, 7) we have assumed that the structure of inositol phospholipide might be of a phosphatidic acid type, inositol being combined with phosphoric acid.

While we were carrying on experiments under this assumption, Faure and Morelec-Coulon (9) described the separation of a new phosphatidic acid from wheat germ which they named glyceroinositolphosphatidic acid, since the hydrolysis of this substance gave 2 moles of fatty acids (saturated and unsaturated), 1 mole of glycerol, 1 mole of phosphoric acid, and 1 mole of inositol. They proposed the accompanying formula as the probable structure of this lipide.

More recently, Hawthorne and Chargaff (10) have taken up this problem and, following the separation methods of Folch and of Carter, have

* Presented before the annual meeting of the Japanese Biochemical Society in Sendai, April, 1954.

XUM



R_1 and R_2 are fatty acid radicals.

isolated the inositol lipide in a relatively pure state and investigated its hydrolysis products. They concluded that the so called inositol lipide they obtained was still contaminated with other compounds and that a glycoside of inositol phosphate may be a contaminant in this lipide. However, they did not elucidate the structure of the inositol-containing lipides. Our purpose was to ascertain the chemical structure of this inositol phospholipide.

We first obtained a crude inositol lipide fraction from commercial soy bean which approached in purity the one Hawthorne and Chargaff had obtained. Next we investigated the combination of inositol in this lipide by methylation studies. A polymethyl ether of inositol was obtained by the hydrolysis of methylated inositol phospholipide.

If the inositol phospholipide from soy bean has a phosphatidic acid type of structure, as we assumed and as Faure and Morelec-Coulon claimed, the methyl ether of inositol obtained by hydrolysis of the methylated lipide should be a pentamethyl inositol. However, if the inositol lipide has more than two ester bonds such as some organic carboxyl group other than phosphoric acid, a tetra or less highly substituted methyl ether of inositol would be obtained by hydrolysis of the methylated lipide. In this communication experiments are reported which were carried out on the above assumption, together with isolation and identification of the fatty acid component of the inositol phospholipide.

EXPERIMENTAL

Analytical Methods—Phosphorus content was determined by the method of Lieb (11); nitrogen, by the micro-Kjeldahl method.

For the determination of the reducing power, a modification of the Hagedorn-Jensen method was used, and the estimation of the methoxyl was accomplished by Vieböck and Brecher's method (12). Inositol was qualitatively measured by Scherer's test, and its content was decided from

the volume of pure inositol obtained by the method of Klenk and Sakai (2) from each fraction of the lipide.

Separation of Crude Inositol Lipide from Commercial Soy Bean Lecithin—1 kilo of commercial soy bean lecithin was extracted in a Soxhlet apparatus with hot acetone and then with methanol. In each case the extraction was continued until the supernatant fluid became colorless. The insoluble residue was dried *in vacuo* (weight, 250 gm.) and dissolved in about 3 volumes of chloroform with vigorous shaking.

To this solution about 1.8 times as much methanol was added and stirred; the cloudy precipitate which appeared was separated by filtration and dried. This procedure was repeated four times. The residue was again dissolved in a minimal amount of chloroform, and this solution was allowed to stand under water for 2 days to remove the proteolipide-like substances (13). The soluble residue, which was obtained after the chloroform layer was separated and evaporated *in vacuo* (weight, 31 gm.), was next treated with boiling dioxane and separated into relatively insoluble (Fraction 1) and relatively soluble (Fraction 2) fractions.

Fraction 1. N 0.8, P 4.1, inositol[1] 4.04, reducing power 2.1
" 2. " 0.6, " 2.9, " 3.66, " " 2.4

The crude inositol lipide thus obtained was a pale yellowish powder, insoluble in water and in common organic solvents; however, it was relatively soluble in wet chloroform and partly soluble in hot dioxane and acetic acid. The yield was about 17 gm. for Fraction 1 and about 9 gm. for Fraction 2.

As Hawthorne and Chargaff demonstrated that both fractions consisted largely of inositol lipide and small amounts of contaminating sugars and nitrogenous compounds, Fraction 1 was subjected to an exhaustive methylation according to Purdie. Fraction 2 was digested with 500 ml. of glacial acetic acid in a boiling water bath for 2 hours with occasional stirring. The insoluble fraction (A) was separated by hot filtration. The precipitate that appeared after solution was cooled was separated by centrifugation (Fraction B), and the soluble fraction (C) was obtained by concentrating the mother liquor *in vacuo* to dryness. The three fractions on analysis gave the following analytical data.

Fraction A. N 0.3, P 2.08, reducing power 7.5, Scherer's test ++
" B. " 0.6, " 3.36, " " 8.6, " " +
" C. " 1.2, " 3.20, " " 7.1, " " ++

We are now continuing the experiment to obtain purer fractions.

[1] Isolated according to the method of Klenk, followed by three more recrystallizations; therefore the true content of inositol should be much higher than the above.

Methylation of Inositol Lipide—Purdie's method of methylation was applied. The procedure was carried out seven times, and once again with half of the methylation reagents: 1 gm. of Fraction 1 was dissolved in 110 ml. of water-saturated chloroform with shaking. To this solution were added 12.5 ml. of methyl iodide, and the whole was put into a flask with a glass-stoppered reflux condenser; after that, 11.6 gm. of freshly precipitated dry silver oxide were added. Then the flask was slightly warmed to accelerate the reaction and left for an hour. Afterward it was kept at mild boiling for 3 hours to complete the reaction. The silver iodide was filtered off and washed thoroughly with chloroform. The filtrate and the washings were collected and concentrated under reduced pressure to about 30 ml. To this concentrate were added about 4 times as much methanol, and the precipitate which appeared was separated by centrifugation. The slightly yellowish supernatant solution was evaporated *in vacuo*, giving a syrupy residue. The methoxyl value of this syrup was 19.8 per cent. The insoluble substances were removed by treating with methanol and ether prior to the determination of methoxyl. Since the precipitate which separated on centrifugation became insoluble even to moist chloroform when dried and its methoxyl content was the same as that of the starting material (2.1 per cent), the authors assumed that it was composed of unchanged contaminants (weight, 2.960 gm.). All of the above syrup (3.65 gm.) was collected, and, as this syrup was soluble in ether, it was again dissolved in 20 ml. of ether and put into a reaction flask to which were added 3.5 ml. of methyl iodide (10 moles) and 6.55 gm. of silver oxide and the mixture was treated as in the first methylation. The silver iodide thus produced was removed by filtration and washed with ether repeatedly. All the ether solutions were combined and evaporated *in vacuo*. The methoxyl value of the syrupy substance so obtained was 24.3 per cent and showed no elevation of methoxyl content after one more methylation.

The authors finally obtained about 3.5 gm. of fully methylated lipide as a syrupy substance which was yellow-colored and almost a paste at room temperature. On warming, it liquefies easily. It is easily soluble in ether and chloroform also, is soluble in ethanol, but insoluble in petroleum ether.

The analytical data of this substance are as follows:

N 0, P 2.1, reducing power 1.5, methoxyl value 24.3

0.29 mg. substance; 5.0 mg. camphor; $\Delta t = 4°$; mol. wt. 580; $[\alpha]_D$ +15° in methanol.

Investigation of the hydrolysis product noted elsewhere in this paper showed it to be hexamethyl glycerophosphoinositide. The same substance was also obtained from Fraction 2 by the same procedure of methylation.

Hydrolysis of Methylated Inositol Lipide—3.0124 gm. of the above substance were dissolved in 10 ml. of absolute methanol; then 40 ml. of absolute methanol containing 10 per cent by weight of hydrochloric acid were added. The solution was put in a flask with a glass-stoppered reflux and boiled for 10 hours on the water bath under an atmosphere of dry carbon dioxide gas. The initial yellow color of the solution turned black rapidly. After 10 hours the flask was cooled overnight in an ice box, and a large amount of methyl ester of fatty acids was precipitated and separated by filtration in the cold. The mother liquor was distilled *in vacuo* and dried. The residue was dissolved in a small amount of methanol and distilled again under reduced pressure to remove the remaining hydrochloric acid. This procedure was repeated three times, after which the residue was emulsified by adding 50 per cent methanol and shaking and heated on the water bath to evaporate the methanol, leaving a watery emulsion. Since traces of methyl esters of fatty acids were present in this emulsion, the emulsion was treated with petroleum ether in a separatory funnel. The layer of petroleum ether was separated and evaporated, and the residue was added to the fatty acid fraction. The remaining water layer was used for the separation of methylated inositol, glycerol, and phosphoric acid respectively.

Fatty Acid Fraction—The crude methyl ester of the fatty acid fraction thus obtained weighed 1.366 gm. It was saponified with 50 per cent methanolic potassium hydroxide. Fatty acids were obtained by evaporation of the methanol and subsequent acidification of the remaining watery solution with 10 per cent sulfuric acid, followed by extraction with ether. The fatty acids were dissolved in about 20 ml. of chloroform, and an excess of bromine was added. At this time the flask was kept cool in running water and left overnight in the cold. After that, the excess bromine and chloroform were removed under reduced pressure. The residue, a mixture of saturated and brominated unsaturated fatty acids, was fractionated by making use of the fact that the brominated unsaturated fatty acid is more soluble in water containing ethanol than is the saturated fatty acid. Therefore the residue was treated with 80 per cent ethanol at $-25°$ to remove the insoluble saturated fatty acid by filtration at the same temperature. The saturated fatty acid fraction obtained by the above procedure was recrystallized repeatedly with 90 per cent ethanol. About 0.6 gm. of snow-white crystals melting at 51–52° was finally obtained.

The analytical results indicated that this substance is myristic acid.

Myristic acid.	Calculated.	Neutralization value 245.81, Ag 32.2	
	Found.	" " 237.91, " (ash from Ag salt) 34.6	

The mother liquor containing brominated unsaturated fatty acid was

evaporated under reduced pressure to remove ethanol, whereby an oily residue was obtained. To remove traces of remaining saturated fatty acid from this oily residue the latter was subjected twice to the procedure described above and treated with a small amount of 80 per cent ethanol at −25°, etc. After these procedures were carried out, the fraction of brominated unsaturated fatty acid was found to contain 28.85 per cent bromine. For the isolation of free unsaturated fatty acid, about 0.2 gm. of this residue was put into a small flask with 2 moles of zinc dust and an absolute methanol solution of 1 mole of hydrochloric acid under reflux and boiled on a water bath for 3 hours to complete the dehalogenation. After that, the zinc dust was filtered off and the traces of hydrochloric acid were also removed by adding barium carbonate and filtration. This methanol solution was distilled *in vacuo* to remove all the methanol, the remaining oily residue was transferred to a distillation apparatus, and the methyl ester of the unsaturated fatty acid was distilled. A colorless oily substance (about 0.1 gm.) was obtained which had the same boiling point as the methyl ester of oleic acid (167°, 1.8 mm. of Hg).

This ester was to be treated for the isolation of the free fatty acid; however, owing to an accident, it was lost.

Methyl Ether of Inositol Fraction—The water layer after the fatty acids had been removed by extraction was extracted with ether for 30 hours. The ether layer was separated and dried over anhydrous sodium sulfate. The dried ether solution was concentrated under reduced pressure, giving 0.3 gm. of oily residue, which was transferred to a small distillation apparatus and fractionated. At 100–101°, 1.8 mm. of Hg, a colorless and odorless liquid weighing about 0.1 gm., were obtained. During this procedure, a distillate of the boiling point coinciding with that of methylated sugar was not obtained.

This liquid has not the boiling point of 133° at 1 mm. of Hg which McGowan (14) described in his methylation studies of inositol; however, the analytical data show that this substance is a pentamethyl inositol.

2.13 mg. substance. Calculated, CH_3O 62.2; found, 60.3

The substance was easily soluble in ethanol, ether, and chloroform, but hard to dissolve in petroleum ether, and its refractive index at 6° was 1.4450. Owing to the small amount, an attempt to prepare an acetyl derivative failed.

Glycerol Fraction—The aqueous layer, after extraction of the methyl ether of inositol, was again concentrated under reduced pressure, and the remaining syrupy residue was treated with a large excess of 20 per cent hydrochloric acid for 15 hours at 110–120° on the oil bath under reflux. During this procedure the contents of the flask were kept at mild boiling

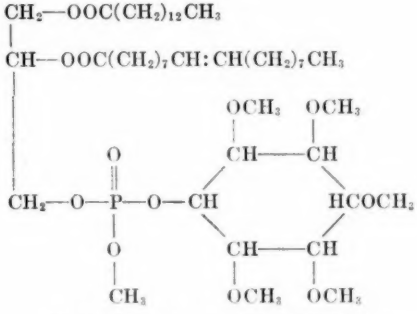
for the purpose of complete hydrolysis of glycerophosphoric acid. After this, the solution was dried under reduced pressure to remove excess hydrochloric acid; then the residue was again dissolved in water. This solution was neutralized with potassium hydroxide until slightly alkaline to litmus. The neutralized solution was evaporated, and to the residue was added absolute ethanol; the flask was then refluxed for an hour on a boiling water bath. The insoluble precipitate was filtered off, and the filtrate was distilled under reduced pressure to remove ethanol. The syrupy residue was transferred to a small distillation apparatus and distilled. A colorless syrup, easily soluble in water and ethanol, insoluble in ether, and having the boiling point of glycerol, 179–185° at 20 mm. of Hg, was obtained in an amount of about 0.08 gm. This substance was further examined by the acrolein test and identified as glycerol.

Phosphoric Acid Fraction—The precipitate which was obtained when glycerol was extracted with ethanol as above was treated by the method of Lieb (11) and precipitated with ammonium molybdate. The precipitate was collected and weighed. The weight of the phosphoric acid was about 0.05 gm.

SUMMARY

The so called inositol phospholipide from commercial soy bean lecithin fraction has been isolated and subjected to Purdie's exhaustive methylation procedure, giving hexamethylglycerophosphoinositide. Hydrolysis of this substance yielded myristic acid,[2] oleic acid, pentamethyl inositol, glycerol, and phosphoric acid.

From these results it may be concluded that the methylated inositol phospholipide which the present authors separated must have the formula illustrated in the accompanying structure.



Hexamethyl glycerophosphoinositide

[2] It is noteworthy that myristic acid was identified as a component of soy bean inositol phospholipide.

Among inositol-containing phospholipides, which many workers up to the present have tried to separate from the fraction of soy bean phospholipides, it is certain that there exists a compound having the structure named by us glycerophosphoinositide which possesses a phosphatidic acid-like structure and which has no nitrogenous base. We hope to continue the investigation of the glycosides of inositol and reducing sugars such as Hawthorne and Chargaff have found in this fraction.

The authors are exceedingly thankful to their director, Professor Morio Yasuda, for his helpful guidance throughout this work. Also they are very grateful to Dr. H. E. Carter of the University of Illinois for various suggestions in preparing this manuscript.

BIBLIOGRAPHY

1. Anderson, R. J., *J. Am. Chem. Soc.*, **52,** 1607 (1930).
2. Klenk, E., and Sakai, R., *Z. physiol. Chem.*, **258,** 33 (1939).
3. Woolley, D. W., *J. Biol. Chem.*, **147,** 581 (1943).
4. Folch, J., and Woolley, D. W., *J. Biol. Chem.*, **142,** 963 (1942).
5. Carter, H. E., and Celmer, W. D., *Physiol. Rev.*, **32,** 167 (1952).
6. Folch, J., *J. Biol. Chem.*, **177,** 497 (1949).
7. Folch, J., *J. Biol. Chem.*, **177,** 505 (1949).
8. Imai, Y., *J. Japan Biochem. Soc.*, **21,** 182 (1949); **25,** 44 (1953).
9. Faure, M., and Morelec-Coulon, M. J., *Compt. rend. Acad.*, **236,** 1104 (1953).
10. Hawthorne, J. N., and Chargaff, E., *J. Biol. Chem.*, **206,** 27 (1954).
11. Lieb, H., *Mikrochemie*, **2,** 78 (1924).
12. Vieböck, F., and Brecher, C., *Ber. chem. Ges.*, **63,** 3207 (1930).
13. Folch, J., and Lees, M., *J. Biol. Chem.*, **191,** 807 (1951).
14. McGowan, J. C., *J. Soc. Chem. Ind.*, **66,** 446 (1947).

THE MECHANISM OF ENZYMIC PHOSPHATE TRANSFER REACTIONS*

By W. H. HARRISON, P. D. BOYER, AND A. B. FALCONE

(From the Department of Agricultural Biochemistry, University of Minnesota, St. Paul, Minnesota)

(Received for publication, October 12, 1954)

Various experimental approaches may yield information to make possible a better understanding of enzymic phosphate transfer. Essential at the outset is a clear recognition of the substrates and cofactors, if any, involved; these have been relatively well defined for many enzymic phosphate transfer reactions. With the exception of Mg^{++}, which appears necessary for all enzymic phosphate transfer reactions studied (1, 2), special cofactor requirements have been demonstrated for only a few phosphate-transferring enzymes, as illustrated by the requirement of pyruvate kinase for K^+ (3) and of phosphoglucomutase for glucose-1,6-diphosphate (4).

Valuable information about phosphate transfer may be obtained with use of isotopes. For example, Meyerhof *et al.* showed that P^{32}-labeled inorganic phosphate was not incorporated into hexose monophosphates during their enzymic interconversion (5), and Jagannathan and Luck showed incorporation of substrate phosphate into phosphoglucomutase (6). The suggestion of the latter studies that an enzyme-phosphate intermediate participates in the catalysis has been supported by later results (7). The value of O^{18} for study of enzymic reactions involving phosphate has been shown by the excellent researches of Cohn (8, 9).

In this paper are presented results of studies with use of O^{18} to determine the fate of phosphate oxygen in enzymic phosphate transfer reactions and in a non-enzymic phosphate transfer reaction together with additional results from study of the pyruvate kinase reaction with pyruvate-2-C^{14}. From these and other studies, postulates about the mechanism of enzymic phosphate transfer are made.

Materials and Methods

Enzyme Preparations—The rabbit muscle extract used for the experiments reported in Table I was prepared by grinding 30 gm. of frozen,

* Taken in part from portions of the thesis of W. H. Harrison for the degree of Doctor of Philosophy. Supported by a research grant from the National Science Foundation and a research grant (1033) from the National Institutes of Health, Public Health Service. Paper No. 3243, Scientific Journal Series, Minnesota Agricultural Experiment Station.

minced rabbit muscle with two 8 ml. portions of cold 0.03 N KOH. The ground mixture was pressed through folded gauze to give a turbid extract.

The acetone powder of a rat muscle extract was prepared as described by Boyer *et al.* (3). The acetone powder of a yeast extract was prepared essentially by the procedure of Hochster and Quastel (10) as described elsewhere (11).

The pyruvate kinase source was the fraction which precipitates at 72 per cent saturation with ammonium sulfate at pH 7.5 in the procedure of Cori *et al.* for the preparation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (12). The acetokinase used was a purified preparation from *Escherichia coli*.[1] The alkaline phosphatase was a commercial preparation (Armour's intestinal phosphatase).

Isotope-Labeled Compounds—Pyruvamide-2-C^{14} was purchased from Tracerlab, Inc., Boston, Massachusetts; the amide was hydrolyzed by allowing a solution to stand for several hours in 0.1 N HCl at room temperature.

Inorganic orthophosphate (P_i) labeled with O^{18} was prepared by careful treatment of P_2O_5 with H_2O^{18} (Stuart Oxygen Company, San Francisco, California) and isolation of the phosphate as KH_2PO_4 was done essentially as described by Cohn (8).

3-Phosphoglycerate labeled with O^{18} in the $-O^-$ oxygens[2] of the phosphate was prepared by the action of a yeast extract on glucose in the presence of P_i-O^{18}. Glucose was converted to hexose diphosphate in accordance with the Harden-Young equation (13) by incubating at room temperature a mixture containing 600 mg. of yeast acetone powder, 8 mg. of diphosphopyridine nucleotide (DPN), 40 μmoles of $MgCl_2$, 20 μmoles of adenosine triphosphate (ATP), 2 mmoles of P_i-O^{18} (0.425 atom per cent excess O^{18}), 3 mmoles of glucose, and 2 mmoles of acetaldehyde in a total volume of 18 ml. at pH 6.2. After 1 hour's incubation no P_i and 1.35 mmoles of fructose-1,6-diphosphate were present. Then 2 ml. of 0.05 M KF and 2 ml. of 0.02 M sodium arsenate, pH 6.2, were added. After an additional 75 minutes incubation, 100 mg. of yeast acetone powder were added; after another 45 minutes incubation 0.26 mmole of fructose-1,6-

[1] The acetokinase was kindly made available for these studies by Professor S. Ochoa and Dr. I. Rose of New York University.

[2] In a compound such as R—O—P(=O)(—O⁻)—O⁻, a principal resonant form of which is R—O—P⁺(—O⁻)(—O⁻)=O, the 3 oxygens not bound to the R group are alike and may be conveniently designated as the $-O^-$ oxygens of the phosphate.

diphosphate remained. The reaction was stopped by the addition of 1 ml. of 30 per cent trichloroacetic acid and the mixture was chilled and centrifuged.

The supernatant solution was neutralized to pH 8.2 with KOH, first with saturated then 1 N KOH. To the solution 25 per cent barium acetate was added carefully until no additional precipitate was formed. The solution was chilled; the precipitate was collected by centrifugation and suspended in 18 ml. of 0.1 N HCl. The undissolved residue was removed by centrifugation and discarded. On standing overnight in a refrigerator, crystals of barium phosphoglycerate formed. The crystals were collected by filtration and dried in a vacuum desiccator; the yield was 101 mg. The crystals contained no inorganic phosphate, fructose-1,6-diphosphate, or easily hydrolyzable phosphate. The total phosphorus present was 9.0 per cent; calculated for $BaOOCCHOHCH_2OPO_3H \cdot 2H_2O$, 8.6 per cent; for the monohydrate, 9.1 per cent.

For determination of the O^{18} content of the phosphate group, 5 ml. of a solution at pH 9.3 containing 59 μmoles of the O^{18}-labeled 3-phosphoglycerate, 0.06 M $MgCl_2$, 0.07 M NH_3-NH_4^+ buffer, and 4 mg. of an intestinal alkaline phosphatase preparation were incubated at 37.5° for 75 minutes. The hydrolysis was 77 per cent complete in this time. The $MgNH_4PO_4$ formed was collected and after addition of carrier inorganic phosphate the O^{18} content was determined as described later. The O^{18} content was equivalent to the presence of 0.244 atom per cent excess O^{18} in the $-O^-$ phosphate oxygens of the original 3-phosphoglycerate. From the mechanism of the enzymic reactions involved, excess O^{18} would be expected only in the $-O^-$ oxygens of the phosphate group and not in the oxygen of the C—O—P linkage. Only the $-O^-$ oxygens would appear in the P_i formed by alkaline phosphatase hydrolysis, because phosphatases catalyze cleavage of the O—P linkage (8).

Alkaline phosphatase catalyzes a slow exchange of phosphate oxygen with that of water (14). To minimize this possibility, the reaction conditions for the above experiment were such as to precipitate the P_i formed as $MgNH_4PO_4$. As a further check, an experiment was run in which P_i labeled with O^{18} replaced the 3-phosphoglycerate; the P_i showed no loss of excess O^{18}.

Isotopic Analyses for C^{14}—The relative activity of preparations containing C^{14} was determined by conventional techniques with an internal Geiger gas flow counter; samples were counted to within 2 per cent standard error. For isolation of phosphopyruvate-2-C^{14} in the presence of large excesses of pyruvate-2-C^{14}, the pyruvate was converted to the 2,4-dinitrophenylhydrazine derivative and the phosphopyruvate (together with other Ba-insoluble phosphates) was isolated as the Ba salt. A typical isolation was as follows: To a 1 ml. enzyme reaction mixture was added 0.5 ml. of 0.01 M

pyruvate (to dilute pyruvate already present) followed by 1 ml. of 0.25 per cent 2,4-dinitrophenylhydrazine in 2 N HCl. The mixture was allowed to stand for 10 minutes at 27°. Then 5 ml. of 95 per cent ethanol were added, the contents were mixed, and 1.5 ml. of 3 M sodium acetate and 0.2 ml. of 0.4 M barium acetate were added. The contents were well mixed, then allowed to stand for 15 to 20 minutes, and the precipitate was collected by centrifugation. The precipitate and centrifuge tube were washed carefully four times with 95 per cent ethanol to remove the alcohol-soluble 2,4-dinitrophenylhydrazone of pyruvate. The samples were then suspended in 0.4 ml. of 95 per cent ethanol saturated with sucrose (the sucrose served to prevent powdering of the dried precipitate) and transferred to a planchet with the aid of small volumes of ethanol. The precipitate was then dried under an infra-red lamp and counted. With the single precipitation procedure as described above phosphopyruvate precipitates were readily obtained which contained less than 0.2 per cent of the pyruvate-2-C^{14} present. By reprecipitation in the presence of additional unlabeled pyruvate, contamination by the pyruvate-2-C^{14} can be reduced to an exceptionally low value.

Isotopic Analyses for O^{18}—For measurement of the O^{18} content, the oxygen of the samples was converted to or equilibrated with carbon dioxide and mass 46/44 ratios were determined with a mass spectrometer.[3]

Inorganic orthophosphate was converted to KH_2PO_4 and heated to drive off H_2O essentially as described by Cohn (8). The O^{18} content of water samples was determined by either of two procedures. For one procedure an intimate mixture of 3.76 gm. of $NaHCO_3$, 0.47 gm. of Na_2SO_3, and 0.19 gm. of $NaHSO_3$ was prepared and dried under vacuum over P_2O_5. A water sample of 20 to 50 mg. in a small stoppered vessel was incubated for 3 hours or more at room temperature with 4.4 mg. of the sulfite-bicarbonate mixture per 50 mg. of water. The sample was then frozen, about 10 mg. of anhydrous $NaHSO_4$ were quickly added, and the tube was evacuated thoroughly while the contents were still frozen. The contents were then thawed to allow the acidic bisulfate to release CO_2.[4] The tube was again immersed in a dry ice-acetone mixture, and the CO_2 was collected for mass spectrometry. Sulfite was used in this procedure because it markedly accelerates the reaction $CO_2 + H_2O \rightleftarrows H_2CO_3$ (15) which limits the exchange of water oxygen with that of CO_2; sulfite and sulfate oxygens do not exchange with oxygen of water under the conditions used (16).

[3] Mass spectrometer analyses were made through the cooperation of Professor A. O. C. Nier and Mr. B. L. Donnally, Jr., of the Department of Physics.

[4] Citric acid is preferable to $NaHSO_4$ as an acidifying agent; with $NaHSO_4$ considerable liberation of SO_2 may occur. The SO_2 does not, however, interfere in the mass 46/44 ratio determinations.

The second procedure was a microadaptation (17) of the method described by Dostrovsky and Klein (18). Water from as little as 25 μmoles of KH_2PO_4 was collected directly in an appropriate vessel containing 0.2 to 0.4 ml. of CO_2 (standard temperature and pressure) and exposed to a hot Pt wire. Exposure for 15 to 20 minutes to a wire heated to a bright yellow color was necessary for complete equilibration. The water was then frozen out and the CO_2 was collected for mass spectrometry.

The 3-phosphoglycerate was isolated from reaction mixtures by barium precipitation following methods as given by Umbreit *et al.* (19) and by Neuberg and Lustig (20). The amorphous barium precipitates obtained in some experiments contained hexose diphosphate and inorganic phosphate as impurities; fortunately, complete removal of these was unnecessary because they did not interfere in the decarboxylation procedure. The small sample sizes made more extensive purification undesirable.

The barium salt was converted to the silver salt essentially as described by Baer and Fischer (21) for preparation of silver phosphopyruvate. The silver salt darkened readily if exposed to light. The silver salt prepared from pure barium 3-phosphoglycerate contained the expected 3 atoms of silver per mole.

For determination of the amount of O^{18} in the carboxyl group of 3-phosphoglycerate 30 to 40 mg. of the silver salt of the acid were decarboxylated by treatment with Br_2 in CCl_4 (22); this reaction has also been used by Little and Bloch for decarboxylation of acetic acid (23). The decarboxylation was carried out in an evacuated system followed by condensation of the Br_2 and CCl_4 in a dry ice-acetone bath (17). The CO_2 formed was transferred to a suitable sample bulb with the aid of a Töpler pump and used directly for mass spectrometer analysis. In Experiments 1 and 2 with rabbit muscle extract (Table I), the CO_2 from the silver salt was collected as $BaCO_3$ by passage into $Ba(OH)_2$. The $BaCO_3$ formed was treated under suitable conditions with concentrated H_2SO_4 to liberate carbon dioxide. This procedure has the disadvantage that slight exchange of oxygen of the carbon dioxide with that of the water of the $Ba(OH)_2$ solution occurs.

The amount of O^{18} in the $—O^-$ oxygens of the phosphate groups of ATP, phosphocreatine, and 3-phosphoglycerate was determined by appropriate hydrolysis to form P_i and the P_i was isolated as $MgNH_4PO_4$. The $MgNH_4PO_4$ was freed of Mg^{++} with a cation exchange column, then neutralized to pH 4.4 to 4.6 with KOH, and KH_2PO_4 was precipitated by addition of alcohol.

To determine the O^{18} content of the phosphate of glycerophosphates, the glycerophosphates were hydrolyzed with alkaline phosphatase by a procedure similar to that described for hydrolysis of 3-phosphoglycerate, except

that the P_i formed was not precipitated until the end of the incubation period.

Miscellaneous Determinations—Inorganic phosphate was determined essentially as described by Sumner (24) and fructose-1,6-diphosphate by the Roe reaction (25). Phosphocreatine phosphate and labile phosphate of ATP were determined by conventional procedures (19).

Results

Incorporation of Phosphate Oxygen into 3-Phosphoglycerate—Considerations of probable enzymic reaction mechanisms suggested that the oxygen required for formation of 3-phosphoglycerate might be derived from inorganic phosphate. To test this possibility, experiments were conducted with rabbit muscle and yeast extracts in which fructose-1,6-diphosphate was cleaved to triose phosphates and the glyceraldehyde-3-phosphate was oxidized with formation of 1,3-diphosphoglycerate in the presence of inorganic phosphate containing O^{18}. Phosphate was transferred to adenosine diphosphate (ADP) by 3-phosphoglycerate kinase present in the extracts and the phosphoglycerate formed was isolated and decarboxylated. The mass 46/44 ratio of the carbon dioxide formed was determined as described under "Materials and methods." The experimental conditions and results are given in Table I.

The results clearly demonstrate that oxygen from inorganic phosphate was present in the carboxyl group of the 3-phosphoglycerate. The amounts of excess O^{18} present in the carboxyl of 3-phosphoglycerate in Experiments 1 and 2 with rabbit muscle were 82.0 and 82.5 per cent of the total possible based on the initial excess O^{18} of the inorganic phosphate and on incorporation of 1 oxygen from phosphate into the carboxyl. The lack of the quantitative incorporation probably resulted principally from slight exchange of the oxygen of CO_2 with water in the isolation procedure used for Experiments 1 and 2 (see "Materials and methods"), as well as from depletion of the excess O^{18} of the inorganic phosphate during the reaction period. With the yeast preparation (Experiment 3) the amount of O^{18} in the carboxyl group was 34 per cent of the total possible based on the initial excess O^{18} of the inorganic phosphate. This apparently low yield was most likely the result of a relatively rapid exchange of inorganic phosphate oxygen with that of water which was catalyzed by the yeast preparation. The results with the muscle and the yeast preparations, together with the very probable high specificity of the enzyme reactions involved towards bonds cleaved, warrant the conclusion that 1 oxygen of the 3-phosphoglycerate carboxyl is derived from phosphate.

Transfer of Labeled Phosphate from 3-Phosphoglycerate to Creatine—In experiments reported previously (26), transfer of phosphate from phospho-

pyruvate to ADP by pyruvate kinase in H_2O^{18} was shown to occur without incorporation of O^{18} into the labile phosphate groups of the ATP formed. These results did not conclusively rule out the possibility that oxygen of the C—O—P bond of the phosphopyruvate may have appeared in the ATP (26). The incorporation of oxygen from substrates, water, or any other source into the phosphate group transferred in an enzymic reaction may be checked by labeling the $—O^-$ oxygens of the phosphate group

TABLE I

Enzymic Incorporation of Oxygen from Inorganic Phosphate into Carboxyl of 3-Phosphoglycerate

The reaction mixture for Experiment 1 contained 1.4 mmoles of Na fructose-1,6-diphosphate, 5.89 mmoles of inorganic phosphate containing 0.364 atom per cent excess O^{18} (5.04 mmoles of added potassium phosphate, 0.85 mmole from enzyme preparation), 4 mmoles of sodium pyruvate, 1 mmole of KF, 0.6 mmole of creatine, and 15 ml. of a rabbit muscle extract in a total volume of 43.0 ml. at pH 7.2. The mixture was incubated for 4 hours at 37°, and then 5 ml. of 30 per cent trichloroacetic acid were added. Conditions for Experiment 2 same as for Experiment 1 except that 6.00 mmoles of phosphate containing 0.365 atom per cent excess O^{18} (5.15 mmoles of potassium phosphate, 0.85 mmole from enzyme preparation) and 1.0 mmole of fructose-1,6-diphosphate were used and the sample was incubated for 1½ hours. The total volume was 39.1 ml. For Experiment 3 the reaction mixture contained 1.96 mmoles of fructose diphosphate, 9.8 mmoles of potassium phosphate (0.425 atom per cent excess O^{18}), 4.9 mmoles of $NaHCO_3$, 10.8 mmoles of acetaldehyde (added in five portions during incubation), 0.74 mmole of KF, and 11 gm. of Fleischmann's dried yeast mixed with 20 ml. of H_2O and 7.4 ml. of CCl_4 in a total volume of 78.8 ml. at pH 6.3. The incubation was for 9 hours at 37.5°.

Experiment No.	Enzyme source	Fructose diphosphate used	Atom per cent excess O^{18} in carboxyl group
		mmoles	
1	Rabbit muscle	1.26	0.150
2	" "	0.95	0.151
3	Yeast	1.33	0.064

with O^{18}. If no loss of O^{18} accompanies the transfer reaction, the reaction must occur without the exchange of the $—O^-$ phosphate oxygens and with cleavage of the O—P bond between the phosphorus and the rest of the donor compound.

The lack of exchange of the $—O^-$ oxygens of phosphate in several enzymic phosphate transfers was demonstrated by use of 3-phosphoglycerate labeled with O^{18} in the $—O^-$ oxygens of the phosphate. In the presence of a suitable muscle extract the phosphate was transferred to creatine; thus the experiment provided a check on possible oxygen exchange reactions accompanying the phosphate transfers of the phosphoglyceromutase, py-

ruvate kinase, and creatine kinase reactions, as well as the enolase reaction. In addition, myokinase and 3-phosphoglycerate kinase were probably present and active in the extract. Phosphocreatine formed was isolated by adding to the reaction mixture 3 ml. of 10 per cent $CaCl_2$ in saturated $Ca(OH)_2$ solution at pH 8.8 to precipitate the inorganic phosphate. The phosphocreatine in the supernatant solution was hydrolyzed by acidification to the equivalent of 1 M HCl and heating at 100° for 7 minutes, and

TABLE II

Transfer of O^{18}-Labeled Phosphate from 3-Phosphoglycerate to Creatine

The reaction mixture for Experiment 1 contained 0.2 mmole of tris(hydroxymethyl)aminomethane, 1 mmole of KCl, 0.1 mmole of $MgCl_2$, 0.010 mmole of ATP, 0.2 mmole of creatine, 0.101 mmole of potassium 3-phosphoglycerate labeled with O^{18}, and 19 mg. of an acetone powder of rat muscle in a total volume of 11.4 ml. at pH 7.2. After incubation for 40 minutes at 37.5°, 3 ml. of 10 per cent $CaCl_2$ in a saturated $Ca(OH)_2$ solution, pH 8.8, were added and the solution was adjusted to pH 8.8 with KOH to precipitate the inorganic phosphate, ATP, and ADP. The amounts of reactants were similar for Experiment 2, except that ATP was replaced by adenylic acid, the amount of potassium 3-phosphoglycerate was 0.09 mmole, the total volume was 8.4 ml., and a dialyzed extract equivalent to 30 mg. of the acetone powder was used. After 40 minutes incubation, another equal portion of the enzyme extract was added, the sample was incubated an additional 40 minutes, and then the $CaCl_2$ solution was added.

Experiment No.	Phosphocreatine formed	Ratio, carrier phosphate to phosphocreatine phosphate	Atom per cent excess O^{18} of $-O^-$ phosphate oxygens	
			3-Phospho-glycerate	Phosphocreatine
	μmoles			
1	76	2.18	0.244	0.234*
2	43	1.84	0.147	0.140

* This value is corrected for dilution by the ATP added and the ATP and phosphocreatine phosphate in the muscle extract; these corrections were not necessary for Experiment 2.

the O^{18} content of the P_i formed was determined. Presence of ATP in the supernatant liquid from calcium precipitation would not interfere because both ATP and phosphocreatine would be expected to give P_i of the same O^{18} content.

The results given in Table II show that, within experimental error, the $-O^-$ oxygens of the phosphate in phosphocreatine had the same atom per cent excess O^{18} as those of 3-phosphoglycerate. Thus the enzymic reactions involved occurred without exchange of the $-O^-$ oxygens of the phosphate group transferred. If 1 oxygen had exchanged per group transferred, the expected atom per cent excess O^{18} in the $-O^-$ oxygens of

phosphocreatine phosphate would have been 0.163 and 0.098 per cent for Experiments 1 and 2, respectively; exchange of 1 atom of oxygen per phosphate was thus readily detectable experimentally.

Transfer of Phosphate from ATP to Acetate in H_2O^{18}—As a test with an additional phosphate transfer reaction, measurements were made with a bacterial acetokinase preparation. Phosphate was transferred from ATP to acetate in H_2O^{18} with hydroxylamine present to react with the acetyl

TABLE III

Transfer of Phosphate from ATP to Acetate in H_2O^{18}

For Experiments 1 and 2, 8 mmoles of potassium acetate, 0.5 mmole of tris(hydroxymethyl)aminomethane, 0.1 mmole of $MgSO_4$, 0.1 mmole of potassium ATP, and 7 mmoles of hydroxylamine hydrochloride were dissolved in H_2O^{18}, then neutralized to pH 7.4 with KOH, and the volume was made up to 10 ml. with H_2O^{18} to give a solution containing 1.27 atom per cent excess O^{18} in the H_2O. A 0.1 ml. aliquot of an acetokinase preparation was added, the sample was incubated for 15 minutes at 29°, and 10 ml. of 10 per cent trichloroacetic acid were added. The inorganic phosphate formed in Experiment 1 (from reaction of the hydroxylamine with acetyl phosphate) was isolated from the supernatant liquid as $MgNH_4PO_4$. In Experiment 2 the ADP, together with residual ATP, was isolated by barium precipitation at pH 8.3, and the product obtained was hydrolyzed in 1 M HCl at 100° to give inorganic phosphate.

Experiment No.	Source of inorganic phosphate	Phosphate transferred	Mass 46/44 ratio of CO_2*	Atom per cent excess O^{18}	
				Observed	Expected for 1 atom of O exchange†
		μmoles			
1	Acetyl phosphate	67.3	0.00416	0.00	0.18
2	ADP‡	86.7	0.00428	0.01	0.15

* The mass 46/44 ratio obtained with control non-labeled phosphate was 0.00419.

† Expected value if 1 atom of oxygen from water appeared in the inorganic phosphate formed, with consideration of the dilution by carrier phosphate.

‡ The ADP fraction isolated contained approximately 13 per cent residual ATP.

phosphate formed, thus giving inorganic phosphate and acethydroxamic acid as final reaction products. The O^{18} contents of the inorganic phosphate derived from the acetyl phosphate and of the easily hydrolyzable phosphate group of ADP were determined. The results given in Table III show that the presence of oxygen from water in the products was equivalent to much less than 1 oxygen per mole of phosphate transferred. These data warrant the conclusion that the phosphate transfer proceeded without exchange of the $—O^-$ oxygens of the phosphate group in a manner analogous to those previously studied.

Acid-Catalyzed Phosphate Transfer in H_2O^{18}—The interconversion of α-

and β-glycerophosphates resulting from heating in strong acid has been regarded as occurring by intermediate formation of a cyclic diester (27). To find out whether this non-enzymic phosphate transfer proceeded in a manner analogous to the enzymic transfer of phosphate, β-glycerophosphate was heated with strong acid in the presence of H_2O^{18}. The experimental conditions and results are given in Table IV. The acidity and heating time exceeded considerably the use of 3.5 N sulfuric acid and ½ hour reflux reported by Chargaff as adequate for conversion of β- into α-glycerophosphoric acid (27). The results show that marked exchange of the phosphate oxygens with those of water had occurred in the refluxed sample. The much smaller exchange noted in the control sample may

TABLE IV

Phosphate Oxygen Exchange during Intramolecular Transfer of Phosphate Catalyzed by Acid

Two solutions containing 7.35 mmoles of β-glycerophosphoric acid in 9 ml. of 4 N HCl with 0.894 atom per cent excess O^{18} in the water were prepared. One solution (control) was allowed to stand at room temperature; the other was refluxed for 3 hours. A second refluxed sample contained inorganic orthophosphoric acid in place of the β-glycerophosphoric acid. Inorganic phosphate formed by slight hydrolysis in the refluxed sample of β-glycerophosphoric acid was removed as $MgNH_4PO_4$, and the O^{18} content of the $—O^-$ oxygens of the phosphate of glycerophosphate was determined by alkaline phosphatase hydrolysis as described in the text.

Source of phosphate analyzed	Mass 46/44 ratio of CO_2	Atom per cent excess O^{18} in phosphate
Control β-glycerophosphate	0.00568	0.08
Refluxed "	0.0131	0.44
" orthophosphate	0.00429	<0.03

have resulted from the known catalysis by alkaline phosphatase of exchange of P_i oxygen with oxygen of water; in the experiments the P_i formed by alkaline phosphatase hydrolysis was not precipitated as $MgNH_4PO_4$ until the end of the incubation period. The data give strong evidence that the phosphate transfer reaction was accompanied by exchange of phosphate oxygen with that of water.

Enzymic Incorporation of Pyruvate-2-C^{14} into Phosphopyruvate—Experiments were conducted to measure the dependence upon various reaction components of the incorporation of pyruvate-2-C^{14} into phosphopyruvate as catalyzed by pyruvate kinase. The data in Table V show the effect of omission of enzyme, ATP and ADP, phosphopyruvate, or K^+ from the complete reaction system. The marked acceleration of the exchange by the addition of ATP and ADP is apparent. The time-course of the dependence of the exchange on the addition of ATP and ADP has been

given in an earlier incomplete report (26). The acceleration of the exchange by addition of phosphopyruvate shows that the radioactivity found in the barium fraction containing phosphopyruvate in the complete system was not the result of net formation of phosphopyruvate from ATP. The effect of added K^+ rules out the possibility that the transfer reaction occurred in two steps, the first of which did not require K^+ and involved transfer of phosphate to an intermediate. The small fraction of the radioactivity of the added pyruvate incorporated into the phosphopyruvate is a reflection of the relatively slow rate of the reaction at equilibrium as contrasted to the rate when phosphopyruvate reacts with ADP in the absence of ATP and pyruvate.

TABLE V

Incorporation of Pyruvate-2-C^{14} into Phosphopyruvate Catalyzed by Pyruvate Kinase

The complete reaction mixture contained in 1.0 ml. total volume, pH 7.4, 0.02 M sodium phosphate buffer, 0.15 M KCl, 0.005 M $MgSO_4$, 0.001 M of a mixture of sodium ATP and ADP (approximately 10 per cent of ADP), 0.001 M sodium pyruvate-2-C^{14} equivalent to approximately 33,000 c.p.m., 0.002 M sodium phosphopyruvate, and rabbit muscle pyruvate kinase. Incubation was for 3 hours at 37°, following which the phosphopyruvate was isolated and its radioactivity determined as described in the text. The pyruvate kinase at 1/100 the concentration used catalyzed the transfer of 0.19 μmole of phosphate from phosphopyruvate to ADP per ml. of reaction mixture in a similar medium without added pyruvate but with added hexokinase and glucose.

Reaction component omitted	Observed radioactivity of phosphopyruvate fraction
	c.p.m.
Enzyme	58
ATP-ADP	63
Phosphopyruvate	115
Potassium	112
None	635

DISCUSSION

The demonstration that an oxygen from phosphate appears in the carboxyl group of 3-phosphoglycerate formed enzymically from glyceraldehyde-3-phosphate shows that the transfer of phosphate from 1,3-diphosphoglycerate to ADP takes place with the cleavage of the O—P bond of the C—O—P linkage. The incorporation of phosphate oxygen into the C—O—P linkage of 1,3-diphosphoglycerate probably results from a nucleophilic displacement by phosphate oxygen on the acyl carbon of a 3-phosphoglyceryl-S-enzyme intermediate formed in the glyceraldehyde-3-phosphate dehydrogenase reaction (28). Such a mechanism is in harmony

with the results of Cohn showing that glyceraldehyde-3-phosphate dehydrogenase does not catalyze exchange of phosphate oxygens with water (9).

The results reported herein also demonstrate that phosphate transfer in the phosphoglyceromutase, pyruvate kinase, and creatine kinase reactions occurs without exchange of the —O⁻ oxygens of the phosphate group transferred with oxygen from any source. Further, they show, that, in the acetokinase reaction, oxygen of phosphate transferred from ATP or of the ADP formed has not exchanged with that of water. Similar results have been obtained with the 3-phosphoglycerate kinase (9) and hexokinase and myokinase reactions.[5] Thus in these enzymic phosphate transfers the —O⁻ oxygens of the phosphate donor remain as such in the phosphate acceptor, and the transfer reactions occur with cleavage of the bond between the P of the phosphate and an O or N of the donor molecule. The phosphate transfer reactions may be regarded as resulting from nucleophilic attack of an O or N of the acceptor molecule on the P atom of the donor (26, 29).

Bücher has presented evidence that the acyl phosphate of 1,3-diphosphoglycerate is bound to the same position on 3-phosphoglycerate kinase as the terminal phosphate of ATP (30); this is in logical agreement with the mechanism suggested by the studies with O^{18}. The phosphate group to be transferred from either phosphate donor may be regarded as being bound at the same site on the enzyme and rendered labile to nucleophilic attack by the acceptor. Additional kinetic studies are necessary to establish whether the concept of single binding sites for the phosphate group transferred is valid for various phosphate-transferring enzymes.

A probable function of Mg^{++} in the catalysis is to combine with the phosphate group to be transferred, thus increasing the residual positive charge on the phosphorus atom and its susceptibility to nucleophilic attack (26). The phosphate group of donor substrates such as phosphopyruvate and phosphocreatine would be expected to have weak affinity for Mg^{++} comparable to that of glucose-1-phosphate (31) and glycerophosphate (32); the enzyme may facilitate combination of the phosphate group to be transferred with Mg^{++}. Present evidence is insufficient to warrant speculation about the manner of such combination.

Although the simplest explanation of the results of the O^{18} studies is the postulate of a direct transfer of phosphate from donor to acceptor, the occurrence of the reaction without exchange of —O⁻ oxygens of the phosphate does not rule out the possibility of formation of enzyme-phosphate intermediates. Formation of such intermediates by the same mechanism

[5] See discussion by M. Cohn in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, p. 520 (1954).

would not lead to exchange of the —O^- oxygens. The studies of the pyruvate kinase reaction with pyruvate-2-C^{14} (Table V and Boyer and Harrison (26)) and of the acetokinase reaction with P^{32} and C^{14} (33) give strong evidence against the formation of enzyme-phosphate intermediates in these enzymic reactions. In contrast, formation of an enzyme-phosphate intermediate in the phosphoglucomutase reaction appears to be well established (7). The phosphate in α-glucose-1-phosphate is on the opposite side of the glucopyranose ring from the phosphate in glucose-6-phosphate; this makes unlikely the direct transfer of phosphate from the 1 to the 6 position by nucleophilic attack of the acceptor oxygen on the phosphorus atom (34). The intermediate formation of an enzyme-phosphate would thus appear logical in the phosphoglucomutase and in other intramolecular phosphate transfers for which similar considerations apply.

Phosphate transfer by phosphatases (35, 36) would be expected to conform to the mechanism suggested above; phosphatases have been shown to catalyze cleavage of O—P bonds and hydrolysis may be regarded as a transfer of the phosphate group to an oxygen of water (29).

The stability of phosphate oxygens in enzymatic phosphate transfer reactions suggests that explanation of the rapid exchange of phosphate oxygen catalyzed by liver mitochondria (9) is to be sought in reactions in which inorganic phosphate participates. Other studies in this laboratory have shown that the uptake of inorganic phosphate accompanying electron transport is readily reversible in liver mitochondria, and the reversal of the initial reactions leading to the uptake of the inorganic phosphate has been suggested as an explanation for the rapid exchange of phosphate oxygen (37).

SUMMARY

Experiments with O^{18} have shown that oxygen from inorganic phosphate appears in the carboxyl group of 3-phosphoglycerate formed by enzymic oxidation of 3-phosphoglyceraldehyde and transfer of phosphate from 1,3-diphosphoglycerate to ADP. The phosphate transfer reaction thus takes place with cleavage of the O—P bond in the C—O—P linkage of 1,3-diphosphoglycerate. Incorporation of phosphate oxygen into the C—O—P linkage suggests that an acyl enzyme formed in the glyceraldehyde-3-phosphate dehydrogenase reaction is cleaved by nucleophilic attack of phosphate oxygen on the acyl carbon.

The enzymic reactions of muscle extract by which the phosphate of 3-phosphoglycerate is transferred to creatine to form phosphocreatine occur without exchange of excess O^{18} originally present in the three —O^- oxygens of 3-phosphoglycerate. The enzymic transfer of phosphate from ATP to acetate by a bacterial enzyme preparation occurred without incor-

with the results of Cohn showing that glyceraldehyde-3-phosphate dehydrogenase does not catalyze exchange of phosphate oxygens with water (9).

The results reported herein also demonstrate that phosphate transfer in the phosphoglyceromutase, pyruvate kinase, and creatine kinase reactions occurs without exchange of the $—O^-$ oxygens of the phosphate group transferred with oxygen from any source. Further, they show, that, in the acetokinase reaction, oxygen of phosphate transferred from ATP or of the ADP formed has not exchanged with that of water. Similar results have been obtained with the 3-phosphoglycerate kinase (9) and hexokinase and myokinase reactions.[5] Thus in these enzymic phosphate transfers the $—O^-$ oxygens of the phosphate donor remain as such in the phosphate acceptor, and the transfer reactions occur with cleavage of the bond between the P of the phosphate and an O or N of the donor molecule. The phosphate transfer reactions may be regarded as resulting from nucleophilic attack of an O or N of the acceptor molecule on the P atom of the donor (26, 29).

Bücher has presented evidence that the acyl phosphate of 1,3-diphosphoglycerate is bound to the same position on 3-phosphoglycerate kinase as the terminal phosphate of ATP (30); this is in logical agreement with the mechanism suggested by the studies with O^{18}. The phosphate group to be transferred from either phosphate donor may be regarded as being bound at the same site on the enzyme and rendered labile to nucleophilic attack by the acceptor. Additional kinetic studies are necessary to establish whether the concept of single binding sites for the phosphate group transferred is valid for various phosphate-transferring enzymes.

A probable function of Mg^{++} in the catalysis is to combine with the phosphate group to be transferred, thus increasing the residual positive charge on the phosphorus atom and its susceptibility to nucleophilic attack (26). The phosphate group of donor substrates such as phosphopyruvate and phosphocreatine would be expected to have weak affinity for Mg^{++} comparable to that of glucose-1-phosphate (31) and glycerophosphate (32); the enzyme may facilitate combination of the phosphate group to be transferred with Mg^{++}. Present evidence is insufficient to warrant speculation about the manner of such combination.

Although the simplest explanation of the results of the O^{18} studies is the postulate of a direct transfer of phosphate from donor to acceptor, the occurrence of the reaction without exchange of $—O^-$ oxygens of the phosphate does not rule out the possibility of formation of enzyme-phosphate intermediates. Formation of such intermediates by the same mechanism

[5] See discussion by M. Cohn in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, p. 520 (1954).

would not lead to exchange of the —O⁻ oxygens. The studies of the pyruvate kinase reaction with pyruvate-2-C^{14} (Table V and Boyer and Harrison (26)) and of the acetokinase reaction with P^{32} and C^{14} (33) give strong evidence against the formation of enzyme-phosphate intermediates in these enzymic reactions. In contrast, formation of an enzyme-phosphate intermediate in the phosphoglucomutase reaction appears to be well established (7). The phosphate in α-glucose-1-phosphate is on the opposite side of the glucopyranose ring from the phosphate in glucose-6-phosphate; this makes unlikely the direct transfer of phosphate from the 1 to the 6 position by nucleophilic attack of the acceptor oxygen on the phosphorus atom (34). The intermediate formation of an enzyme-phosphate would thus appear logical in the phosphoglucomutase and in other intramolecular phosphate transfers for which similar considerations apply.

Phosphate transfer by phosphatases (35, 36) would be expected to conform to the mechanism suggested above; phosphatases have been shown to catalyze cleavage of O—P bonds and hydrolysis may be regarded as a transfer of the phosphate group to an oxygen of water (29).

The stability of phosphate oxygens in enzymatic phosphate transfer reactions suggests that explanation of the rapid exchange of phosphate oxygen catalyzed by liver mitochondria (9) is to be sought in reactions in which inorganic phosphate participates. Other studies in this laboratory have shown that the uptake of inorganic phosphate accompanying electron transport is readily reversible in liver mitochondria, and the reversal of the initial reactions leading to the uptake of the inorganic phosphate has been suggested as an explanation for the rapid exchange of phosphate oxygen (37).

SUMMARY

Experiments with O^{18} have shown that oxygen from inorganic phosphate appears in the carboxyl group of 3-phosphoglycerate formed by enzymic oxidation of 3-phosphoglyceraldehyde and transfer of phosphate from 1,3-diphosphoglycerate to ADP. The phosphate transfer reaction thus takes place with cleavage of the O—P bond in the C—O—P linkage of 1,3-diphosphoglycerate. Incorporation of phosphate oxygen into the C—O—P linkage suggests that an acyl enzyme formed in the glyceraldehyde-3-phosphate dehydrogenase reaction is cleaved by nucleophilic attack of phosphate oxygen on the acyl carbon.

The enzymic reactions of muscle extract by which the phosphate of 3-phosphoglycerate is transferred to creatine to form phosphocreatine occur without exchange of excess O^{18} originally present in the three —O⁻ oxygens of 3-phosphoglycerate. The enzymic transfer of phosphate from ATP to acetate by a bacterial enzyme preparation occurred without incor-

poration of O^{18} from $HO^{18}H$ into the ADP or acetyl phosphate formed. These results show conclusively that, in the reactions catalyzed by phosphoglyceromutase, pyruvate kinase, creatine kinase, and acetokinase, the three $-O^-$ oxygens of the phosphate donor remain as such in the phosphate acceptor and that the transfer reactions occur with cleavage of the bond between the P of phosphate and an O or N of the donor molecule. Also the data demonstrate that the enolase reaction proceeds without any exchange of phosphate oxygen.

The enzymic phosphate transfer reactions studied and other similar phosphate transfer reactions are regarded as proceeding by nucleophilic attack of an O or N atom in the acceptor molecule on the P atom of the donor phosphate compound. The results do not rule out possible formation of enzyme-phosphate intermediates. Additional implications of the data are discussed.

In contrast to the enzymic transfer, treatment of β-glycerophosphate with acid under conditions which cause intramolecular migration of the phosphate group results in incorporation of O^{18} from $HO^{18}H$ into the phosphate group. This gives support to the diester mechanism proposed for such phosphate transfer.

The incorporation of pyruvate-2-C^{14} into phosphopyruvate in the presence of pyruvate kinase is dependent upon the presence of K^+ as well as ADP. This is evidence against the formation of an enzyme-phosphate intermediate in the pyruvate kinase reaction.

BIBLIOGRAPHY

1. Lardy, H. A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 447 (1951).
2. Colowick, S. P., in Sumner, J. B., and Myrbäck, K., The enzymes, New York, **2,** pt. 1, 114 (1951).
3. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149,** 529 (1943).
4. Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F., and Trucco, R. E., *Arch. Biochem.*, **22,** 87 (1949).
5. Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, H., *Biochem. Z.*, **298,** 296 (1938).
6. Jagannathan, V., and Luck, J. M., *J. Biol. Chem.*, **179,** 569 (1949).
7. Najjar, V. A., and Pullman, M. E., *Science*, **119,** 631 (1954).
8. Cohn, M., *J. Biol. Chem.*, **180,** 771 (1949).
9. Cohn, M., *J. Biol. Chem.*, **201,** 735 (1953).
10. Hoehster, R. M., and Quastel, J. H., *Arch. Biochem. and Biophys.*, **31,** 278 (1951).
11. Robertson, H. E., and Boyer, P. D., *J. Biol. Chem.*, **214,** 295 (1955).
12. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173,** 605 (1948).
13. Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, **80,** 299 (1908).
14. Stein, S. S., and Koshland, D. E., Jr., *Arch. Biochem. and Biophys.*, **39,** 229 (1952).
15. Roughton, F. J. W., and Booth, V. H., *Biochem. J.*, **32,** 2049 (1938).
16. Mills, G. A., *J. Am. Chem. Soc.*, **62,** 2833 (1940).
17. Harrison, W. H., Thesis, University of Minnesota (1954).

18. Dostrovsky, I., and Klein, F. S., *Anal. Chem.*, **24,** 414 (1952).
19. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis (1949).
20. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1,** 311 (1942–43).
21. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **180,** 145 (1949).
22. Hunsdiecker, H., and Hunsdiecker, C., *Ber. chem. Ges.*, **75,** 291 (1942).
23. Little, H. N., and Bloch, K., *J. Biol. Chem.*, **183,** 33 (1950).
24. Sumner, J. B., *Science*, **100,** 413 (1944).
25. Roe, J. H., *J. Biol. Chem.*, **107,** 15 (1934).
26. Boyer, P. D., and Harrison, W. H., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 658 (1954).
27. Chargaff, E., *J. Biol. Chem.*, **144,** 455 (1942).
28. Segal, H. L., and Boyer, P. D., *J. Biol. Chem.*, **204,** 265 (1953).
29. Koshland, D. E., Jr., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 608 (1954).
30. Bücher, T., *Biochim. et biophys. acta*, **1,** 292 (1947).
31. Trevelyan, W. E., Mann, P. F. E., and Harrison, J. S., *Arch. Biochem. and Biophys.*, **39,** 440 (1952).
32. Tabor, H., and Hastings, A. B., *J. Biol. Chem.*, **148,** 627 (1943).
33. Rose, I., Grunberg-Manago, M., Korey, S., and Ochoa, S., *Federation Proc.*, **13,** 283 (1954).
34. Lardy, H. A., in Respiratory enzymes, Minneapolis, 185 (1949).
35. Green, H., and Meyerhof, O., *J. Biol. Chem.*, **197,** 347 (1952).
36. Axelrod, B., *J. Biol. Chem.*, **172,** 1 (1948).
37. Boyer, P. D., Falcone, A. B., and Harrison, W. H., *Nature*, **174,** 401 (1954).

Rec
have s
for *Ac*
clude t
In enz
(4) ha
synthe
lacking
of vari
of the
Sinc
probal
approx
a com
might
ations
compo
to thi
Diagra
has be

Syn
(*a*) pe

* Th
Naval
stitute
Commi
partme
Charle
gon St
† Pr
Frankf

XUM

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF PANTETHEINE ANALOGUES*

XVI. PANTOTHENIC ACID STUDIES

By CHARLES J. STEWART,† VERNON H. CHELDELIN, and TSOO E. KING

(From the Department of Chemistry and the Science Research Institute, Oregon State College, Corvallis, Oregon)

(Received for publication, December 18, 1954)

Recent reports from Brown and Snell (1, 2) and from this laboratory (3) have shown that *N*-pantothenylcysteine has high growth-stimulatory effect for *Acetobacter suboxydans*. This fact has led the former workers to conclude that pantothenylcysteine is the immediate precursor of pantetheine. In enzymatic biosynthesis of coenzyme A (CoA), Hoagland and Novelli (4) have confirmed Brown and Snell's idea. However, the details of the synthesis as well as the knowledge of patterns in other organisms are still lacking. A study of the behavior of pantetheine analogues in the growth of various organisms would yield valuable information about the synthesis of the coenzyme *in vivo*.

Since CoA is a relatively large nucleotide, its structural analogues will probably be extremely difficult to synthesize chemically. The most facile approach to a study of inhibitory analogues would probably be to produce a compound that could block the biosynthesis of the coenzyme or that might even be transformed *in vivo* into a CoA analogue. By proper alterations in the structure of pantetheine, it should be possible to prepare compounds which would have this action. The present paper is a preface to this method of attack. Eight analogues of pantetheine (as shown in Diagram 1) have been prepared, and their effect upon microbial growth has been studied.

EXPERIMENTAL

Synthetic Methods—Several known compounds were prepared as follows: (*a*) performic acid by adding 2 ml. of 30 per cent hydrogen peroxide to

* This investigation was supported in part by research grants from the Office of Naval Research and the Division of Research Grants and Fellowships, National Institutes of Health. Published with the approval of the Monographs Publications Committee, Oregon State College, Research paper No. 267, School of Science, Department of Chemistry. A portion of this work was submitted by one of the authors, Charles J. Stewart, in partial fulfilment of the degree of Doctor of Philosophy, Oregon State College, 1955.

† Present address, Department of Organic Chemistry, University of Frankfurt, Frankfurt, Germany.

XUM

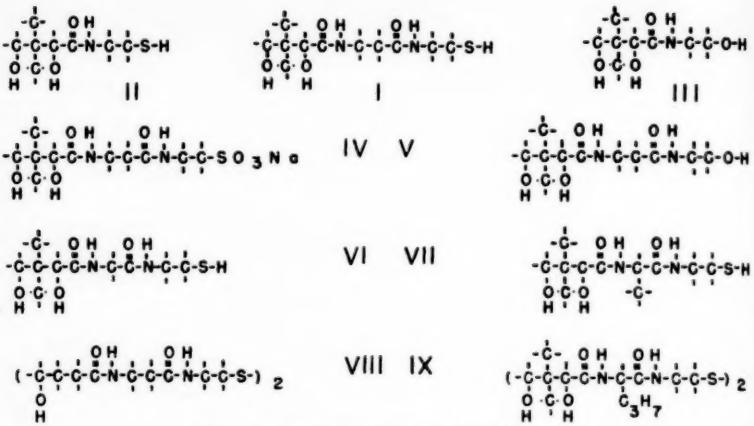


DIAGRAM 1. Pantetheine and analogues

18 ml. of 90 per cent formic acid (5); (*b*) carbobenzoxy chloride according to the directions of Bergmann and Zervas (6); (*c*) 2-aminoethanethiol as described by Mills and Bogert (7); (*d*) β-aletheine and (*e*) pantetheine (I, Diagram 1), by the method of King, Stewart, and Cheldelin (8); (*f*) *N*-pantoylethanolamine (III) by fusion of pantolactone and 2-aminoethanethiol according to Barnett (9).

Pantothenyltaurine-Sodium Salt (IV)

The sodium salt of pantetheine was prepared by dissolving 1.95 gm. of crude pantetheine (obtained as a fusion mixture) in 4 ml. of 1.118 N sodium methoxide. This mixture was added to 20 ml. of performic acid. After standing at room temperature for 15 minutes, the reaction solution was poured into 200 ml. of acetone. To facilitate precipitation of sodium formate, approximately 50 ml. of ether were added to the acetone solution. The turbid mixture was left at room temperature for 3 days. The solid was centrifuged off and the supernatant liquid concentrated to dryness at 1 mm. pressure. Traces of formic acid in this residue were removed by redissolving in absolute ethanol and again concentrating to dryness at 1 mm. pressure. The residue was dissolved in about 5 ml. of methanol and poured into ether. The resultant solid gave a qualitative test for sulfur and nitrogen. The carbon, hydrogen, and ash determinations indicated purity of about 80 per cent.

A precipitate formed during the final removal of ethanol. Advantage was taken of this phenomenon in purification by dissolving the crude salt of pantothenyltaurine in a small volume of ethanol, centrifuging off the insoluble sodium formate, and pouring the clear supernatant solution into

ether. The solid formed was redissolved in methanol and precipitated by ether. The process was repeated twice. 100 mg. of a white powder were formed, which softened at 102°,[1] formed a semimolten mass at 104°, and effervesced on further heating.

$C_{11}H_{21}NaN_2O_7S$. Calculated, C 37.92, H 6.08; found,[2] C 38.31, H 6.28

Bis(N-(γ-hydroxybutyryl)-β-alanyl-2-aminoethyl) Disulfide (VIII)[3]

500 mg. of β-aletheine (8) were heated together with 350 mg. of γ-butyrolactone at 100° for 10 minutes in order to melt the β-aletheine. Then the material was fused at 70–80° for 1 hour. The fusion mixture was dissolved in 1 ml. of methanol and permitted to stand overnight for oxidation by air to occur. The material which crystallized out was redissolved by adding 2 ml. of methanol. About 3 ml. of ether were then added to incipient turbidity. After 2 days the crystals were centrifuged off and recrystallized successively from absolute ethanol, isopropyl alcohol, and finally from absolute ethanol. Yield, 100 mg. of yellowish white crystals; m.p. 196.5–198°.

$C_{18}H_{34}N_4O_6S_2$. Calculated, C 46.32, H 7.34, N 12.00; found, C 46.33, H 7.31, N 12.12

"Oxypantetheine" (N-Pantothenylethanolamine) (V)

Carbobenzoxy-β-alanine—The synthesis by Sifferd and du Vigneaud (11) was employed, with the exception that pure β-alanine rather than succinimide was the starting material.

Carbobenzoxy-β-alanyl Chloride—The method of Dyer and Ballard (12) was used to prepare an ether solution of this compound, which was immediately used in the next step of the synthesis.

N-(Carbobenzoxy-β-alanyl)ethanolamine—5 gm. of sodium bicarbonate (0.06 mole) and 2.8 gm. of ethanolamine (0.046 mole) were dissolved in 40 ml. of water and cooled in a salt-ice bath to 0–5°. To the cold solution, 5 ml. portions of a 50 ml. ether solution of carbobenzoxy-β-alanyl chloride, prepared from 10 gm. of carbobenzoxy-β-alanine (0.045 mole), were added slowly with stirring. The temperature was maintained at 0–5° at all times. The product precipitated from the cold reaction mixture. Stirring was continued for 15 minutes after all the acid chloride had been added. The mixture was filtered and the product air-dried overnight. The dry

[1] All melting points were obtained on a Fisher melting point block and are reported as uncorrected.

[2] Chemical analyses were performed by the Weiler and Strauss Microanalytical Laboratory, Oxford, England, and by Micro-Tech Laboratories, Skokie, Illinois.

[3] While this work was in progress, preparation of this compound was reported by Bowman and Cavalla (10).

crude product was crystallized from chloroform. Yield, 6 gm.; m.p. 119–121°.

Further purification was obtained by recrystallization from ethyl acetate. Yield, 4 gm.; m.p. 126–126.5°.

$C_{13}H_{18}N_2O_4$. Calculated, C 58.63, H 6.81, N 10.5; found, C 58.48, H 6.43, N 11.2

N-β-Alanylethanolamine Oxalate—7 gm. of carbobenzoxy-β-alanylethanolamine (0.025 mole) were added to about 100 ml. of anhydrous liquid ammonia. Small pieces of metallic sodium (1.3 gm. or 0.056 mole) were added until a blue color persisted for 10 minutes. 3.5 gm. of ammonium chloride (0.06 mole) were then added and the liquid ammonia was permitted to boil to dryness at room temperature. The free base was separated from sodium chloride by extraction with 300 ml. of 95 per cent ethanol. The traces of ammonia in the alcohol solution were removed by concentrating to dryness under reduced pressure (1 mm.). The residual oil was redissolved in 30 ml. of absolute ethanol and added to 3 gm. of oxalic acid dihydrate dissolved in about 30 ml. of isopropyl alcohol. The initial precipitate of the oxalate was redissolved in heating in order to obtain uniform crystallization. Ether was added to incipient turbidity and the solution placed at −4° for crystallization. The product appeared to contain alcohol of crystallization. It melted at 70–80° without drying, but, when dried overnight at 1 mm. pressure over P_2O_5, the melting point rose to 118–121°. Yield, 3.1 gm. After recrystallization from absolute ethanol, it melted at 122–123°.

$C_7H_{14}N_2O_6$. Calculated, C 37.83, H 6.35; found, C 37.77, H 6.36

"Oxypantetheine" (N-Pantothenylethanolamine)[4]—888 mg. of the above oxalate salt (4 mmoles) were dissolved in 15 ml. of absolute methanol, and to this were added 7.38 ml. of 1.0813 N sodium methoxide solution. The sodium oxalate was removed by centrifugation and washed once with a small volume of methanol. The combined washing and supernatant fluid were concentrated to dryness under a vacuum (1 mm.) to yield a white powder. The powder was transferred to a tube, 600 mg. (4.6 mmoles) of pantolactone[5] were added, and the tube was sealed. The sealed tube was heated at 65–70° for 4 hours.

Purification was attempted by two plate counter-current extraction with a solvent system of butanol-acetic acid-water (4:1:5). The fusion product was dissolved in 10 ml. of the upper phase, extracted with 10 ml. of the

[4] After this work had been completed, a paper by Baddiley and Mathias (13) appeared on the synthesis of "oxypantetheine."

[5] The natural (−) isomer of pantolactone was used throughout the course of this work.

lower phase, and transferred for reextraction with a fresh 10 ml. portion of the lower phase. The lower phase used in the first extraction was extracted with a fresh 10 ml. portion of the upper phase, which was in turn reextracted by the second lower phase. The combined upper phases were frozen in an attempt to remove the excess pantolactone by sublimation under a high vacuum. A highly viscous hygroscopic sirup was obtained in this manner.

Analysis for the total nitrogen and pantolactone content (14) indicated a purity of 85 per cent.

$C_{11}H_{22}N_2O_5$. Calculated. Pantolactone 49.6, C 50.36, H 8.45, N 10.68
Found. " 57.3, " 50.98, " 8.55, " 8.17

N-(Pantoylglycyl)-2-aminoethanethiol (VI)

Carbobenzoxyglycine and *carbobenzoxyglycyl chloride* were prepared according to the method of Bergmann and Zervas (6).

Bis(N-(carbobenzoxyglycyl)-2-aminoethyl) Disulfide[6]—3.4 gm. of 2-aminoethanethiol were dissolved in 40 ml. of water and cooled to 0°. Hydrogen peroxide was added dropwise until a positive nitroprusside reaction (without cyanide) was no longer given by a drop of the solution. Sodium hydroxide (6 N) was added until the mixture was alkaline to phenolphthalein. 50 ml. of an ethereal solution of carbobenzoxyglycyl chloride, prepared from 10 gm. of carbobenzoxyglycine, were added in portions in a salt-ice bath with constant stirring and cooling. Throughout the course of the reaction the solution was maintained alkaline to phenolphthalein by addition of sodium hydroxide, and the temperature was kept below 5°. After all the acid chloride had been added, stirring was continued for 10 minutes, and then the product which had risen to the surface was removed by filtration, washed with water, and air-dried overnight. 3 gm. were obtained after recrystallization from 95 per cent ethanol; m.p. 175–178°.

$C_{24}H_{30}N_4O_6S_2$. Calculated, N 10.5; found, N 10.8

N-Glycyl-2-aminoethanethiol—3.8 gm. of bis(*N*-carbobenzoxyglycylaminoethyl) disulfide were suspended in approximately 50 ml. of liquid ammonia in a 3-necked flask. The flask was fitted with a soda lime tube for exclusion of carbon dioxide and moisture and with a stirring blade. The ammonia suspension of the carbobenzoxy compound was vigorously stirred, while a total of 1.0 gm. of sodium was added in small portions until the blue color of excess sodium persisted for 10 minutes. 2.5 gm. of ammonium chloride were added, after which the solution was left at room temperature until the ammonia had evaporated. The residue was extracted with alco-

[6] One sample of this intermediate was kindly furnished by H. J. Sharkey of this laboratory.

hol and the sodium chloride removed by filtration and washed with alcohol. 2.5 gm. of oxalic acid dihydrate were dissolved in about 25 ml. of absolute alcohol and added to the combined filtrate and washings.

A small amount of ammonium oxalate which formed was removed by filtration after heating the solution on a boiling water bath to dissolve any precipitated glycylaminoethanethiol oxalate. To the clear warm filtrate, ether was added to incipient turbidity. Crystallization occurred at −4°. Yield, 1 gm. The product melted with decomposition at 165–167°.

$C_6H_{12}N_2O_5S$. Calculated, N 12.49; found, N 12.42

N-(Pantoylglycyl)-2-aminoethanethiol—448 mg. of glycylaminoethanethiol oxalate were dissolved in dry methanol. An equivalent amount of sodium methoxide was added (4 m.eq.). Sodium oxalate was removed by centrifugation and washed once with 1 ml. of dry methanol. The supernatant fluid and washings were concentrated at 1 mm. pressure to a small volume and transferred to a test-tube narrowed at the top for sealing. The methanol solution was then evaporated to dryness *in vacuo* over P_2O_5. 300 mg. of pantoyl lactone were added to the dry material. The tube was sealed and the contents were fused in a glycerol bath at 65–70° for 4 hours.

Purification was attempted through use of counter-current extraction in the butanol-acetic acid-water system. The fusion product was dissolved in 10 ml. of the upper layer and carried through twelve plates in an all-glass Craig counter-current distribution apparatus. Plates 4 to 8 inclusive, which exhibited a positive nitroprusside and negative ninhydrin test, were concentrated to dryness. Data on nitrogen analysis indicated a purity of about 80 per cent. Analysis (14) revealed pantolactone to be the major contaminant.

$C_{10}H_{20}N_2O_4S$.	Calculated.	C 45.43, H 7.62, N 10.59, S 12.13,	pantolactone 49.3
	Found.	" 46.51, " 7.71, " 8.24, " 10.28,	" 53.4

N-(Pantoylalanyl)-2-aminoethanethiol (VII)

Phthalylalanine—10 gm. of DL-alanine were fused with 16.7 gm. of phthalic anhydride for 30 minutes at 170–180°, according to Beilstein (15). Crystallization was effected by dissolving the fusion mixture in approximately 100 ml. of boiling ethanol and adding water to the first appearance of turbidity. 18.4 gm. comprised the first crop of crystals, with a melting point of 162.5–164°. Concentration of the mother liquor yielded an additional 4.3 gm. for a total yield of 22.7 gm. Beilstein (15) reports a melting point of 160–162°.

Phthalylalanyl chloride was prepared by suspending 15 gm. of phthalylalanine in 25 ml. of thionyl chloride and heating at 40° until a homogeneous

solution was formed. The bulk of the unchanged thionyl chloride was removed *in vacuo*, and traces remaining in the solid residue were removed by adding chloroform and concentrating to dryness. The white solid residue was not purified further. It was dissolved in ether and used immediately in the next step of the synthesis.

Bis(N-(phthalylalanyl)-2-aminoethyl) Disulfide—3.5 gm. of 2-aminoethanethiol were added to 40 ml. of water at 0–5°. The solution was oxidized by the dropwise addition of hydrogen peroxide with stirring until a negative nitroprusside was obtained without added cyanide. 5.5 gm. of sodium bicarbonate were dissolved in the mixture. Stirring and chilling were continued while small portions of the ether solution of phthalylalanyl chloride, prepared from 10 gm. of phthalylalanine, were poured into the reaction vessel. 15 minutes after all the acid chloride had been added, stirring was discontinued and the product was removed by filtration and air-dried. This material contained traces of salts and had a melting point range of 219–225°. Recrystallization was achieved through solution in boiling pyridine and addition of ethanol to incipient turbidity; a recrystallized sample melted at 217–221°.

$C_{26}H_{26}N_4O_6S_2$. Calculated, C 56.30, H 4.72, N 10.10; found, C 56.25, H 4.92, N 9.76

N-Alanyl-2-aminoethanethiol Oxalate—11.1 gm. (0.02 mole) of the foregoing phthalyl compound were suspended in 120 ml. of absolute ethanol, and 40 ml. of 1 M hydrazine hydrate in ethanol were added. The reaction mixture was refluxed for 1.5 hours. The undissolved solid was separated by filtration and the clear supernatant liquid concentrated at 1 mm. pressure. After removal of ethanol, the residue was combined with the solid obtained by filtration and suspended in 30 ml. of 2 N HCl. The acid suspension was heated for 5 minutes at 53° to extract all acid-soluble material, and then placed at room temperature for 100 minutes. Solid phthalyl hydrazide (5.8 gm.) was removed by filtration and the clear filtrate was vacuum-dried from the frozen state.

The solid left after removal of all water was dissolved in about 100 ml. of liquid ammonia. 2.8 gm. of sodium were added, with constant stirring, to the ammonia solution. Complete reduction was indicated by the blue color of excess sodium persisting for several minutes. 7 gm. of ammonium chloride were then added to the reaction vessels and the ammonia was permitted to boil to dryness. The dry residue was extracted with 50 ml. of absolute ethanol and the sodium chloride (9 gm.) was filtered off. Traces of ammonia were removed from the clear extract by evaporation to dryness at 1 mm. pressure. The residue was dissolved in 30 ml. of ethanol and poured into 20 ml. of ethanol containing 2.6 gm. of oxalic acid dihydrate. No precipitate formed on cooling. Addition of 75 to 100 ml. of ether pre-

cipitated a brown hygroscopic oil. The ether-ethanol was decanted and more ether was added to this clear liquid until the solution was slightly turbid. Fine needle-like crystals formed after standing at room temperature for several days. These were removed by filtration. Yield, 2 gm.; m.p. 141–143°. This material exhibited positive ninhydrin and nitroprusside tests (without cyanide), but failed to yield the correct analytical results.

N-(Pantoylalanyl)-2-aminoethanethiol—1.08 gm. of the foregoing oxalate salt were dissolved in 15 ml. of dry methanol. To this solution were added 8.4 ml. of 1.0813 N sodium methoxide in dry methanol. The sodium oxalate which precipitated was separated by centrifugation. The clear supernatant solution was decanted and concentrated at 1 mm. pressure. As the concentration proceeded, more solid precipitated from the methanol solution, which would not redissolve in methanol. When a volume of about 2 ml. was reached, the solution and the rinses from the flask were transferred to a test-tube with a constricted neck for ease of sealing. Evaporation at 1 mm. pressure over P_2O_5 was resumed until a dry residue remained in the tube. 650 mg. of pantolactone were placed in the tube. The tube was sealed and the mixture was placed in a glycerol bath at 85–90° for 4 hours for the ensuing fusion reaction.

Purification was attempted by dissolving the fusion mixture in 10 ml. of the upper phase of a butanol-acetic acid-water system (4:1:5) and subjecting it to a sixteen plate counter-current extraction. Plates 9 to 15 inclusive exhibited a positive nitroprusside reaction with added cyanide, but failed to show a ninhydrin test, indicating that these tubes contained no starting material. The respective phases from these plates were combined. The combined (water) phases were washed once with 20 ml. of fresh upper (butanol) phase. The combined upper phases and washings were vacuum-dried from the frozen state. The viscous sirupy residue thus obtained was redissolved in water and again vacuum-dried from the frozen state to remove traces of solvent. A purity of about 80 per cent was obtained by this procedure, as shown by the following analysis:

$C_{11}H_{22}N_2O_4S$.	Calculated.	C 47.45, H 7.96, N 10.06, S 11.51
	Found.	" 49.52, " 7.93, " 8.03, " 9.87

Mercuric Mercaptide of Alanine Analogue—The combined water phases from the counter-current extraction Plates 9 to 15 were also lyophilized. The sirupy residue was dissolved in 5 ml. of water and stirred for 20 minutes with 200 mg. of mercuric oxide. The unchanged mercuric oxide was removed by centrifugation, and the clear water solution was lyophilized. The solid remaining after removal of water was dissolved in 4 ml. of butanol, and 12 ml. of acetone were added. After standing overnight at

−4°, the solution was centrifuged and the clear mother liquor removed by decantation. The solid was dried over P_2O_5 at room temperature and 1 mm. pressure overnight. A purity of 88.5 per cent was indicated by elemental analysis, as carbon, hydrogen, and nitrogen were approximately 12.5 per cent below the expected values.

$C_{22}H_{42}N_4O_8S_2Hg$. Calculated. C 34.98, H 5.61, N 7.42, Hg 26.6, pantolactone 34.4
Found. " 30.89, " 4.93, " 6.52, " 31.1(16), pantolactone 32.5

Bis(N-(pantoylnorvalyl)-2-aminoethyl) Disulfide (IX)

Phthalylnorvaline—10 gm. of DL-norvaline were fused with phthalic anhydride at 170–180° for 30 minutes. At the end of this period the molten mass was poured into about 80 ml. of benzene. Undissolved material was removed by filtration. Petroleum ether (b.p. 36°) was added to the clear benzene until incipient turbidity was obtained. Fine needle crystals formed overnight, which were separated from the mother liquor by filtration. Yield, 18 gm.; m.p. 103–104°.

$C_{13}H_{13}NO_4$. Calculated, C 63.14, H 5.30, N 5.66; found, C 63.10, H 4.88, N 5.52

Phthalylnorvalyl Chloride—15 gm. of phthalylnorvaline were suspended in approximately 30 ml. of thionyl chloride. This suspension mixture was heated at 40° to final solution of solids and termination of gas evolution. The excess thionyl chloride was removed by evaporation at 1 mm. pressure. The oily residue was dissolved in a small amount of chloroform and again concentrated to dryness to remove traces of thionyl chloride. The oily residue was not further purified but was dissolved in about 60 ml. of ether for immediate use in the next step of the synthesis.

Bis(N-(phthalylnorvalyl)-2-aminoethyl) Disulfide—4.7 gm. of 2-aminoethanethiol were dissolved in 75 ml. of H_2O. The solution was chilled to 0–5° in a salt-ice bath. Hydrogen peroxide (30 per cent) was added dropwise, with continual stirring, until complete oxidation to the disulfide was indicated by failure to give a positive sulfhydryl test with sodium nitroprusside in the absence of cyanide. 10 gm. of sodium bicarbonate were then added and 5 to 10 ml. portions of the foregoing ethereal solution of phthalylnorvalyl chloride were added to the reaction mixture. Vigorous stirring and chilling were maintained so that the temperature remained at 0–5° throughout the course of the reaction. 10 minutes after all the acid chloride solution had been added, the solid product was separated by filtration and air-dried. Purification was acquired by recrystallization from ethanol. Yield, 7 gm.; m.p. 173–175°.

$C_{26}H_{24}N_4O_6S_2$. Calculated, C 58.99, H 5.61, N 9.17; found, C 58.39, H 5.34, N 9.35

Bis(N-norvalyl-2-aminoethyl) Disulfide Dihydrochloride—6.1 gm. (0.01

mole) of the intermediate just described were suspended in 40 ml. of absolute ethanol. 20 ml. of 1 N ethanolic hydrazine hydrate were added and the mixture was refluxed. Within the first 15 minutes complete solution was observed, followed by slow formation of a precipitate. At the end of 2 hours, the solution was cooled to room temperature. The solid was filtered from the solution and set aside for later extraction with acid. The clear solution was concentrated to dryness under reduced pressure. The filtered solid was added to the residue from the supernatant solution and the combined solids were suspended in 40 ml. of 1 N hydrochloric acid. The acidified suspension was heated at 50° for 10 minutes and then cooled to room temperature. The phthalyl hydrazide that formed (3.1 gm.) was removed by filtration. The clear filtrate was concentrated to dryness from the frozen state. Lyophilization was continued overnight to remove all traces of moisture. The dihydrochloride was dissolved in 20 ml. of absolute ethanol. Ether was added until the solution became cloudy, then placed in an acetone-dry ice bath overnight. After warming to room temperature in a desiccator, the clear supernatant liquid was decanted from the solid. The white crystalline product was washed with ether. Yield, 2 gm. The material started to decompose at 160°.

$C_{14}H_{28}N_4O_2S_2Cl_2$.	Calculated.	C 40.18, H 6.72, N 13.36, S 15.36
	Found.	" 39.02, " 7.44, " 13.4, " 15.3

Bis(N-(pantoylnorvalyl)-2-aminoethyl) Disulfide—1.06 gm. of the above dihydrochloride salt were dissolved in methanol. An equivalent amount of sodium methoxide in methanol was added. Any precipitated sodium chloride was removed by centrifugation. The supernatant liquid was concentrated to dryness at 1 mm. pressure. Pantolactone (700 mg.) was added to the residual sirup and fused (sealed tube) at 85–90° for 8 hours. Purification was attempted by subjecting the fusion mixture to a twenty-six plate counter-current extraction in butanol-acetic acid-water (4:1:5). The solutions in Plates 21 to 25, which gave a negative ninhydrin reaction, were concentrated to dryness. Paper chromatography indicated the presence of only one sulfur-containing component. Since the elementary analysis did not check with the calculated values, the pantoyl lactone content was determined. The calculated value is 42.5 per cent, and that found was 46.4 per cent. The compound thus appeared to be about 93 per cent pure.

Microbiological Methods—Lactobacillus helveticus 80 (kindly supplied by Dr. E. E. Snell) and *Lactobacillus bulgaricus* ATCC 8001 were carried on Difco litmus milk. *Lactobacillus fermenti* ATCC 9338 was carried on agar stabs, the composition of which was 0.5 per cent glucose, 1 per cent yeast extract, 0.5 per cent peptonized milk, and 2 per cent agar. *A. suboxydans*

ATCC 621 was maintained on slants of medium containing 0.5 per cent yeast extract, 5 per cent glycerol, and 2 per cent agar.

The microbiological assays were conducted as described by Craig and Snell (17) for lactobacilli, and by Sarett and Cheldelin (18) for *A. suboxydans*. Lactobacilli inocula were grown at 37° for 22 to 24 hours on 10 ml. of the basal medium supplemented with either 100 γ of pantetheine or 100 γ of calcium pantothenate. *Acetobacter* inocula were obtained from a 30 to 40 hour culture grown at 30° in a 50 ml. Erlenmeyer flask in 10 ml. of basal medium to which 100 γ of pantetheine had been added.

The amount of bacterial growth was determined turbidimetrically on a Pfaltz and Bauer fluorophotometer and expressed in terms of optical density (2 log per cent light transmission).

Snell and Shive (19) report that *N*-pantoylethanolamine (*cf.* Diagram 1, Compound II) competitively inhibits the growth of certain pantothenic acid-requiring bacteria, *e.g. Leuconostoc mesenteroides* P-60. However, Table I demonstrates that for a pantetheine-requiring organism such as *L. helveticus*, II as well as *N*-pantoyl-2-aminoethanethiol (III) is biologically inert.

Despite the importance of the thiol group for enzymatic activity in the CoA family of compounds, one is led to speculate that biological activity in pantetheine and CoA is also dependent upon two amide bonds in the molecular structure, probably as points of attachment to the protein. Such attachment might easily be effected through hydrogen bonding. The inactivity of *N*-methylpantothenic acid reported earlier (20) is in line with this idea.

Replacement of the β-alanine fragment of pantetheine with either glycine or α-alanine produces analogues with lowered growth-sustaining ability. Table II demonstrates the growth stimulation of *L. helveticus* by the glycine and α-alanine analogues (*N*-(pantylglycyl)-2-aminoethanethiol (VI) and *N*-(pantoylalanyl)-2-aminoethanethiol (VII)). Residual pantolactone, if any, contained in these preparations has no effect on the microbial growth as witnessed in Table IV.

Substitution of glycine for the β-alanine moiety in pantetheine decreases the activity to about 0.02 per cent of an equal weight of pantetheine. It is apparent that the distance between the two amide groups is of importance for active utilization, presumably in order to secure attachment to the CoA-synthesizing enzymes.

Introduction of a methyl group onto the methylene carbon in the glycine analogue, as in the α-alanine analogue, causes no improvement in biological activity. The α-alanine analogue possesses only about 0.01 per cent of the activity of pantetheine, which is of the same order of magnitude as the glycine compound. However, when a bulky group is introduced onto this

methylene carbon, as in the norvaline analogue (IX), biological activity is lost altogether. Presumably, IX is incapable of attachment to the receptor enzymes due to the steric hindrance of the large propyl group.

TABLE I

Growth Response of L. helveticus 80 to Pantetheine, N-Pantoyl-2-aminoethanethiol (III), and N-Pantoylethanolamine (V)

Pantetheine analogue, γ per 10 ml.		Pantetheine, γ per 10 ml.		
		0	15	20
		Optical density		
Pantoylaminoethanethiol	0	0.01	0.47	0.51
	100	0.02	0.47	0.51
	1000	0.02	0.51	0.56
Pantoylethanolamine	0	0.02	0.40	0.52
	100	0.02	0.40	0.52
	1000	0.02	0.43	0.57

TABLE II

Growth Stimulation of L. helveticus by N-(Pantoylglycyl)-2-aminoethanethiol (VI), N-(Pantoylalanyl)-2-aminoethanethiol (VII), and Bis(N-(γ-hydroxybutyryl)-β-alanyl-2-aminoethyl) Disulfide (VIII)

Amount added	Pantetheine analogue	Growth response, pantetheine equivalent	Activity of analogue
γ		γ	*per cent*
50	Glycyl	0.012	0.024
100	"	0.028	0.028
250	"	0.072	0.029
500	"	0.092	0.018
1000	"	0.138	0.014
50	α-Alanyl	0.006	0.012
100	"	0.009	0.009
250	"	0.015	0.006
500	"	0.030	0.006
1000	"	0.069	0.007
100	γ-Hydroxybutyryl	0.075	0.075
250	"	0.173	0.069
500	"	0.32	0.064

The pantoic acid moiety of pantetheine also contributes to the configurational requirements of the molecule, as might be expected from earlier studies with analogues of the free vitamin (21). The α-hydroxybutyric acid analogue (VIII) is only 0.07 per cent as active (*cf.* Table III) for *L.*

helveticus and completely inactive for *A. suboxydans*, which requires the pantoic acid fragment for growth.

TABLE III

Effect of Pantolactone on Growth of L. helveticus in Presence of Pantetheine

Pantolactone, γ per 10 ml.	Pantetheine, γ per 10 ml.			
	0	0.05	0.10	0.15
	Optical density			
0	0.02	0.05	0.22	0.29
250	0.03	0.05	0.21	0.30
1000	0.04	0.05	0.20	0.30

TABLE IV

Growth Stimulation of L. helveticus by Pantothenyltaurine (IV)

Pantetheine, γ per 10 ml.	Pantothenyltaurine, γ per 10 ml.			
	0	5	10	15
	Optical density			
0	0.05	0.18	0.31	0.41
0.1	0.30	0.37	0.41	0.49

TABLE V

Growth Stimulation of L. fermenti by Pantothenyltaurine (IV)

Pantothenyltaurine	Growth response, pantetheine equivalent	Relative activity
γ per 10 ml.	*γ*	*per cent*
0	0	
10	0.045	0.45
20	0.055	0.28
30	0.08	0.27
40	0.16	0.40
Average		0.35

The unusual structure of pantoic acid confers not only unique biological properties upon its derivatives, but also characteristic physical behavior. All compounds containing this residue in their structure are hygroscopic and tend to form viscous sirups, whereas the γ-hydroxybutyryl analogue is obtained easily as a stable crystalline compound. It is possible that the

α-hydroxy groups which have also been shown previously (21) to be necessary for attachment to enzyme surfaces may provide a means for hydrogen bonding.

TABLE VI

Inhibition of L. helveticus by Oxypantetheine (V)

Pantetheine, γ per 10 ml.	Oxypantetheine, γ per 10 ml.				
	0	5	10	25	100
	Optical density				
0	0.03	0.03	0.03	0.03	0.03
0.10	0.25	0.41	0.20	0.04	0.02
0.15	0.34	0.54	0.36	0.06	0.04
0.25	0.50	0.64	0.57	0.29	0.05

TABLE VII

Competitive Inhibition of L. helveticus by Oxypantetheine (V)

Pantetheine	Oxypantetheine	$\frac{\text{Oxypantetheine}}{\text{Pantetheine}}$	Optical density
γ per 10 ml.	*γ per 10 ml.*		
0	0	0	0.11
0.05	0	0	0.34
0.05	5	100	0.54
0.05	7.5	150	0.47
0.05	10	200	0.38
0.10	0	0	0.49
0.10	10	100	0.52
0.10	15	150	0.44
0.10	20	200	0.36
0.15	0	0	0.53
0.15	15	100	0.52
0.15	22.5	150	0.47
0.15	30	200	0.36
0.25	0	0	0.60
0.25	25	100	0.53
0.25	37.5	150	0.45
0.25	50	200	0.38

Oxidation of the sulfhydryl group of pantetheine to a sulfonic acid produces a compound (IV), as shown in Table IV, with 1 per cent of the activity of pantetheine. This slight growth-promoting activity is in marked contrast to the behavior of pantoyltaurine, as previously observed in most lactic acid bacteria. This analogue of the free vitamin is a competitive

inhibitor of pantothenate in many microorganisms as well as plant and animal systems. The failure of IV to inhibit growth of *L. helveticus* is perhaps due to the hydrolysis of IV by the organism to free pantothenic acid; the latter is about 1 per cent as active as pantetheine for this organism (17). Pantothenyltaurine is also capable of supporting the growth of *L. fermenti*, as shown in Table V. This growth may be also due to partial hydrolysis of pantothenyltaurine to free pantothenic acid, since *L. fermenti* responds to the free vitamin rather than pantetheine. *A. suboxydans* has previously been shown to hydrolyze pantoyltaurine (21).

Replacement of the SH group by OH forms "oxypantetheine" (V), an active competitive inhibitor for *L. helveticus* (*cf.* Tables VI and VII) with an inhibition index (inhibitor : substrate ratio) of 100 to 200 at half maximal growth. Wieland *et al.* (22) have shown that *S*-phenylpantetheine is

TABLE VIII

Stimulation of Growth of L. fermenti by Oxypantetheine (V)

Oxypantetheine	Growth response, calcium pantothenate equivalent	Activity
γ per 10 ml.	*γ*	*per cent*
10	0.014	0.14
20	0.049	0.25
60	0.164	0.27
100	0.270	0.27
Average		0.23

also an active competitive inhibitor, while the *S*-methyl and *S*-ethyl derivatives are slowly hydrolyzed to pantetheine.

The mode of action of "oxypantetheine," as well as *S*-phenylpantetheine, is probably due to formation of a CoA analogue which competes with CoA for the enzyme surface. This seems plausible on the basis of the structural features of V, which include an unaltered pantothenyl residue (hydroxy and amide groups and carbon skeleton duplicating those in the vitamin). On the other hand, substitution of the thiol group by a hydroxy group evidently prevents the formation of S ~acyl bonds, which are characteristic products of CoA activity. Presumably an altered CoA may be formed, which competes with CoA in normal transacylation reactions.

The synergistic effect that low concentrations (*cf.* Table VI, 5 γ level) of "oxypantetheine" exert with pantetheine is more difficult to explain. Craig and Snell (17) have shown pantothenic acid to have similar synergistic action with pantetheine in *L. helveticus*. Table VIII demonstrates

XUM

that some hydrolysis can occur. "Oxypantetheine" has a pantothenic acid activity of 0.23 per cent for *L. fermenti*. Whether the growth may result from pantothenic acid arising from hydrolysis, or a competition of "oxypantetheine" for pantetheine-hydrolyzing enzymes, thus preventing some normal partial destruction of the vitamin, cannot be determined from the present studies.

SUMMARY

1. Eight new analogues of β-aletheine and pantetheine have been synthesized as listed in Diagram 1.

2. *N*-Pantoyl-2-aminoethanethiol, *N*-pantoylethanolamine, and bis(*N*-pantoylnorvalyl-2-aminoethyl) disulfide were unable to stimulate the growth of *Lactobacillus helveticus*.

3. *N*-(Pantoylglycyl)-2-aminoethanethiol, *N*-(pantoylalanyl)-2-aminoethanethiol, and bis(*N*-(γ-hydroxybutyryl)-β-alanylaminoethyl) disulfide were able to support the growth of *L. helveticus*, a pantetheine-responsive organism, at a concentration of 100 to 1000 γ per 10 ml. of culture medium.

4. *N*-Pantothenyltaurine was able to stimulate the growth of both *Lactobacillus fermenti* and *L. helveticus* at concentrations of 5 to 60 γ per 10 ml. of culture.

5. *N*-Pantothenylethanolamine ("oxypantetheine") was an active, competitive inhibitor of pantetheine in *L. helveticus* with an inhibition index of 100 to 200 at 50 per cent inhibition. A concentration of "oxypantetheine" of at least 10 γ per 10 ml. of culture was required to support the growth of *L. fermenti*.

BIBLIOGRAPHY

1. Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, **75,** 2782 (1953).
2. Brown, G. M., and Snell, E. E., *J. Bact.*, **67,** 465 (1954).
3. King, T. E., and Cheldelin, V. H., *Proc. Soc. Exp. Biol. and Med.*, **84,** 591 (1953).
4. Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.*, **207,** 767 (1954).
5. Toennies, G., and Homiller, R. P., *J. Am. Chem. Soc.*, **64,** 3054 (1942).
6. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65,** 1192 (1932).
7. Mills, E. J., Jr., and Bogert, M. T., *J. Am. Chem. Soc.*, **62,** 1173 (1940).
8. King, T. E., Stewart, C. J., and Cheldelin, V. H., *J. Am. Chem. Soc.*, **75,** 1290 (1953).
9. Barnett, J., *J. Chem. Soc.*, 5 (1944).
10. Bowman, R. E., and Cavalla, J. F., *J. Chem. Soc.*, 1171 (1954).
11. Sifferd, R. H., and du Vigneaud, V., *J. Biol. Chem.*, **108,** 753 (1935).
12. Dyer, E., and Ballard, E., *J. Am. Chem. Soc.*, **59,** 1697 (1937).
13. Baddiley, J., and Mathias, A. P., *J. Chem. Soc.*, 2803 (1954).
14. Szalkowski, C. R., and Davidson, J. H., Jr., *Anal. Chem.*, **25,** 1192 (1953).
15. Prager, B., and Jacobson, P., Beilsteins Handbuch der organischen Chemie, Berlin, 4th edition, **21,** 482 (1935).
16. Irving, H., Andrew, G., and Risdon, E. J., *J. Chem. Soc.*, 541 (1949).

17. Craig
18. Saret
19. Snell
20. Lind
21. Chel
22. Wiel

XUM

17. Craig, J. A., and Snell, E. E., *J. Bact.*, **61,** 283 (1951).
18. Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **159,** 311 (1945).
19. Snell, E. E., and Shive, W., *J. Biol. Chem.*, **158,** 551 (1945).
20. Lindsay, R. D., and Cheldelin, V. H., *J. Am. Chem. Soc.*, **72,** 828 (1950).
21. Cheldelin, V. H., and Schink, C. A., *J. Am. Chem. Soc.*, **69,** 2625 (1947).
22. Wieland, T., Möller, E. F., and Dieckelmann, G., *Chem. Ber.*, **85,** 1035 (1952).

(*From t*
Instit

The
(5HTA
biosynt
for the
stances
designe
scribed
in the
2-naph

Mat
Compa
Reag
Bora
added
n-buta
shakin
permit
n-Bu
equal
finally
Hep
the *n*-
1-N
cent et
Nitr
NaNO
Proc
extrac

* Pr

XUM

THE ESTIMATION OF 5-HYDROXYTRYPTAMINE (SEROTONIN) IN BIOLOGICAL TISSUES

By SIDNEY UDENFRIEND, HERBERT WEISSBACH, AND CARROLL T. CLARK*

(From the Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland)

(Received for publication, December 22, 1954)

The following methods for the estimation of 5-hydroxytryptamine (5HTA) in tissues were developed to make possible various studies on its biosynthesis, metabolism, and physiological disposition. The procedure for the extraction of 5HTA from tissues to separate it from interfering substances is common to all the methods. Three methods for assay of 5HTA designed to meet different situations of sensitivity and specificity are described in this paper. These are absorption in the ultraviolet, fluorescence in the ultraviolet, and colorimetric estimation after reaction with 1-nitroso-2-naphthol.

EXPERIMENTAL

Materials—5-Hydroxytryptamine was made available by The Upjohn Company and the Abbott Laboratories as the creatinine sulfate complex.

Reagents—

Borate buffer. To 94.2 gm. of boric acid dissolved in 3 liters of water are added 165 ml. of 10 N NaOH. The buffer solution is then saturated with *n*-butanol (see below) and NaCl by adding these substances in excess and shaking. Excess *n*-butanol is removed by aspiration and excess salt is permitted to settle. The final pH should be approximately 10.

n-Butanol. Reagent grade butanol is purified by shaking first with an equal volume of 0.1 N NaOH, then with an equal volume of 0.1 N HCl, and finally twice with distilled water.

Heptane. Practical grade of heptane is treated in the same manner as the *n*-butanol.

1-Nitroso-2-naphthol reagent. 0.1 per cent 1-nitroso-2-naphthol in 95 per cent ethyl alcohol.

Nitrous acid reagent. To 5 ml. of 2 N H_2SO_4 is added 0.2 ml. of 2.5 per cent $NaNO_2$. The reagent should be prepared fresh daily.

Procedure for Extraction of 5HTA from Tissue Extracts—5HTA can be extracted into *n*-butanol from salt-saturated solutions buffered to pH 10.

* Present address, Department of Chemistry, University of Virginia.

XUM

By washing the *n*-butanol several times with salt-saturated buffer of pH 10, substances such as 5-hydroxytryptophan, tryptophan, and normally occurring "blank" impurities can be removed. Addition of heptane to the *n*-butanol makes it possible to reextract the 5HTA into aqueous acid, in which it can be assayed. 3 ml. of tissue extract are adjusted to approximately pH 10 and transferred to a 60 ml. glass-stoppered bottle containing 1 ml. of the borate buffer, 2 gm. of NaCl, and 15 ml. of *n*-butanol. The bottle is shaken for 5 minutes. The contents are decanted into a 45 ml. glass-stoppered centrifuge tube and centrifuged. The aqueous phase is removed by aspiration, 15 ml. of borate buffer reagent are added, and the tube is shaken for 5 minutes. After allowing the phases to separate, the aqueous layer is removed, replaced with 15 ml. of fresh buffer reagent, shaken for 5 minutes, and centrifuged. A 10 ml. aliquot of the butanol layer is transferred to another 45 ml. glass-stoppered centrifuge tube containing 20 ml. of heptane and 3 ml. of 0.5 M formate buffer of pH 4.[1] The tube is shaken, centrifuged, and the supernatant solvent layer is removed by aspiration. The aqueous layer containing the extracted 5HTA is assayed by one of the three methods presented below.

The distribution of 5HTA between the salt-saturated buffer, pH 10, and *n*-butanol is such that with the volumes used only about 95 per cent is extracted into the organic solvent. Since three equilibrations of the butanol with salt-saturated buffer are employed, the final recovery of 5HTA is about 85 per cent. Standards are therefore prepared by carrying known amounts of 5HTA through the entire extraction procedure.

The extraction procedure yields extracts which are essentially free of any interfering material, within the limits of sensitivity found for each of the following analytical procedures. The extraction procedure also removes compounds such as tryptophan and 5-hydroxytryptophan when they are used as substrates for the production of 5HTA.

Assay by Ultraviolet Absorption

The absorption spectrum of 5HTA in acid solution shows a maximum at 275 mμ and a subsidiary peak at approximately 295 mμ. Studies involving formation or destruction of 5HTA by liver or kidney slices or homogenates can be followed by extracting 5HTA from the incubation mixture by the above procedure and then measuring the absorption at 275 mμ. This method has been applied to studies of the activities of 5-hydroxytryptophan decarboxylase (1) and monoamine oxidase in a number of tissues. It is applicable to the determination of 0.04 to 0.4 μmole of 5HTA in as much as 500 mg. of tissue. Optical density is proportional to

[1] When the colorimetric procedure is to be employed, 0.2 M HCl is used instead of formate buffer.

concentration to about 0.4 μmole per ml. when measured in a 1 cm. cell in the Beckman spectrophotometer. Above this concentration 5HTA shows marked deviations from Beer's law.

In experiments with 5-hydroxytryptophan decarboxylase relatively large amounts of 5HTA are formed. The only substances which could contribute to ultraviolet absorption are 5-hydroxytryptophan or substances which may be derived from it other than by decarboxylation to 5HTA. 5-Hydroxytryptophan is completely removed during the course of the extraction process. In a previous report (1) it was demonstrated that 5HTA is the only product of the action of a partially purified 5-hydroxytryptophan decarboxylase preparation on its substrate. This was shown by paper chromatography, ultraviolet absorption spectra, and comparison of distribution coefficients of the apparent product with those of authentic 5HTA. It was also shown that for each mole of 5-hydroxytryptamine formed 1 mole of 5-hydroxytryptophan disappeared and 1 mole of CO_2 was generated. The spectrophotometric and colorimetric procedures yielded similar values in 5-hydroxytryptophan decarboxylase studies.

Colorimetric Procedure

1-Nitroso-2-naphthol reacts with many substituted phenols in strong nitric acid to yield colored derivatives. This reaction has been widely used for qualitative (2) and quantitative (3) assay of tyrosine. 5-Hydroxyindoles also react with 1-nitroso-2-naphthol, but can do so in the absence of HNO_3. In dilute HCl or H_2SO_4 containing traces of nitrite they yield violet chromophores. In the absence of nitric acid tyrosine does not react with the reagent. In fact, of over twenty phenolic compounds tested *p*-hydroxyacetanilide was the only compound not a 5-hydroxyindole which reacted to give a violet color. For example, tryptophan, tryptamine, 7-hydroxytryptophan, and 7-hydroxytryptamine do not give this color.

The absorption spectrum of the 5HTA chromophore is presented in Fig. 1. The same spectra are obtained with 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, and *N*-dimethyl-5-hydroxytryptamine.

To 2 ml. of the acid extract containing 0.05 to 0.8 μmole of 5HTA in a glass-stoppered centrifuge tube is added 1 ml. each of the nitrosonaphthol and the acid nitrite reagents. The tube is stoppered, shaken, and placed in a water bath at 55° for 5 minutes. 10 ml. of ethylene dichloride are added, and the tube is shaken to extract the unchanged nitrosonaphthol. The tube is then centrifuged at low speed, and the supernatant aqueous layer is transferred to a cuvette. When measured at 540 mμ in a Beckman spectrophotometer, optical density is proportional to concentration up to 0.8 μmole. The color is stable, less than 1 per cent change occurring on standing 1 hour.

The colorimetric procedure has approximately the same sensitivity as the ultraviolet absorption technique. It is the method of choice when tissue extracts are found to contain large amounts of ultraviolet-absorbing material.

The nitrosonaphthol reagent can also be applied as a spray on paper chromatograms. The paper is first sprayed with 0.1 per cent nitrosonaphthol reagent. After drying, it is sprayed with the nitrous acid reagent.[2] 5-Hydroxyindoles appear as violet spots on a faint yellow background. Extracts of platelets and of rabbit stomach and intestine yield one violet

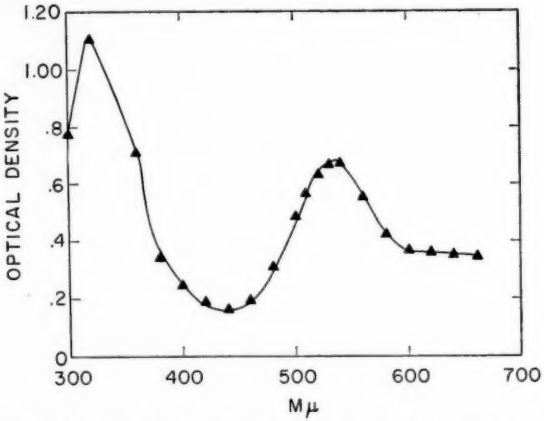


FIG. 1. Absorption spectrum of chromophore obtained by reaction of 5-hydroxytryptamine with 1-nitroso-2-naphthol. To 80 γ of 5HTA in 2 ml. of dilute acid was added 1 ml. each of nitrosonaphthol and acid nitrite reagents. The sample was incubated and extracted with ethylene dichloride as described above.

spot having the R_F of 5HTA on chromatograms developed with *n*-propanol-1 N NH_3 (5:1) or *n*-propanol-1 N HCl (5:1) and then sprayed with these reagents. In some cases a very small amount of a more organic soluble basic 5-hydroxyindole could be detected.

Fluorometric Procedure

In humans the concentration of 5HTA in the blood is approximately 0.1 to 0.3 γ per ml., practically all of the material being found in the platelets (4, 5). Such small concentrations cannot be detected by either the colorimetric or spectrophotometric procedures unless huge amounts of blood are used. The development of a spectrophotofluorometer capable

[2] When used as a spray for chromatography, the nitrous acid reagent is made with HCl instead of H_2SO_4.

of activating compounds and measuring their emitted fluorescence continuously from 250 to 650 mμ revealed that 5HTA fluoresces in the ultraviolet (6). The intensity of the emitted fluorescence is sufficient to permit measurement of 5HTA on 5 to 10 ml. samples of blood.

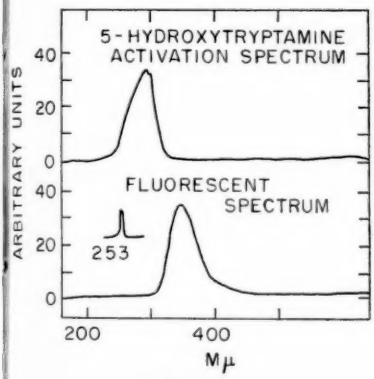


Fig. 2

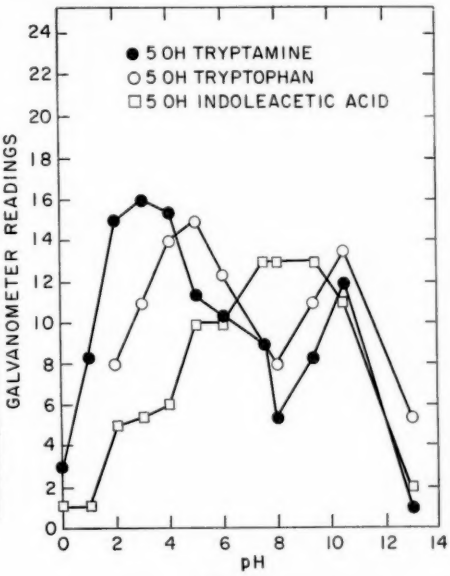


Fig. 3

Fig. 2. Activation and fluorescence spectra of 5-hydroxytryptamine. To obtain the activation spectrum the fluorescence monochromator was set to the maximum for 5HTA (330 mμ), and the spectrum from the activating monochromator was scanned. To obtain the fluorescence spectrum the activating monochromator was set to the maximum for 5HTA (295 mμ), and the spectrum from the fluorescence monochromator was scanned. The 253 mμ peak is one of the mercury lines used for calibration.

Fig. 3. Variations in intensity of fluorescence of some 5-hydroxyindoles with changing pH. The following solutions were used to obtain the indicated pH: pH 0, 1 M H_2SO_4; pH 1, 0.1 M H_2SO_4; pH 2, 0.01 M H_2SO_4; pH 3, 0.1 M acetic acid; pH 4, 0.1 M acetate; pH 5, 0.1 M acetate; pH 6, 0.1 M phosphate; pH 7, 0.1 M phosphate; pH 8, 0.1 M phosphate; pH 9.5, 0.25 M pyrophosphate; pH 10.5, 0.2 M tris(hydroxymethyl)-aminomethane; pH 13, 0.1 M NaOH.

Spectrophotofluorometer—The apparatus is described in detail elsewhere (6). It consists of a xenon arc as a continuous light source and two quartz monochromators at right angles, one to select the activation energy and one to select the emitted fluorescence. An ultraviolet-sensitive photo-

multiplier scanning device coupled to an oscillograph or automatic recorder produces records of the spectra which can be used both for identification and quantitative analysis.

Fluorescent Properties of 5-Hydroxytryptamine—Activation of 5HTA occurs maximally at 295 mμ, coinciding with one of the peaks in its absorption spectrum. The maximum of the fluorescence spectrum occurs at 330 mμ (Fig. 2). The final solution in the extraction procedure is adjusted to pH 4.0 to obtain maximal fluorescence (Fig. 3).

Procedure for Fluorometric Estimation of 5HTA in Platelets—Platelets are isolated from 5 to 10 ml. of human blood according to the procedure of

TABLE I

Recovery of 5-Hydroxytryptamine from Platelets

Species	5HTA added	5HTA found	Recovery*
	γ	γ	*per cent*
Human		1.70	
	0.70	2.35	98
	0.70	2.45	102
	1.40	2.96	95
	1.40	3.07	99
Dog		4.17	
	1.40	5.55	101
	1.40	5.60	102
	2.80	6.70	97
	2.80	7.22	105

* Platelets were isolated from about 100 ml. of blood and suspended in Versene-saline solution (7). Aliquot portions of the platelet suspension, equivalent to approximately 7 ml. of original blood, were used in each assay.

Dillard, Brecher, and Cronkite (7). A platelet count is taken on a sample of the original blood and on the saline suspension of isolated platelets to correct for loss of platelets during isolation. An aliquot of the isolated platelet suspension, containing 0.05 to 3.0 γ of 5HTA, is carried through the extraction procedure as previously described, except that the volume of the final buffer solution at pH 4.0 is 1.5 ml. instead of 3.0 ml. Fluorescence is measured in the spectrophotofluorometer and compared to standards carried through the entire procedure.

5HTA when added to platelet suspensions can be recovered quantitatively (Table I). Fluorescence is directly proportional to concentration over the entire range given. The fluorophor obtained from platelets by this procedure exhibits exactly the same activation and fluorescence spectra as does pure 5HTA (Fig. 4). Extracts obtained from large amounts of platelets yielded the same value for 5HTA by all three methods of assay.

Fluorescence shows more discrimination than colorimetric or spectrophotometric assay. All the 5-hydroxyindoles examined have exactly the same absorption spectrum and give exactly the same color with nitrosonaphthol. They are all activated to fluoresce with light of the same wavelength, 295 mμ, and emit the same fluorescent light, 330 mμ. However, each compound has its own optimal conditions of pH for maximal fluorescence (Fig. 3). It is therefore possible to distinguish between various 5-hydroxyindole derivatives by selecting the proper pH in which to make the fluorescence measurements. An additional advantage of the recording

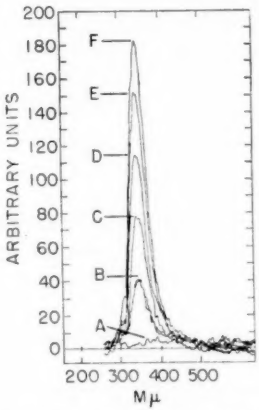


FIG. 4. Fluorescence spectra of 5-hydroxytryptamine from blood platelets and of standards carried through the extraction procedure. *A* = blank, *B* = 0.7 γ of 5HTA, *C* = 1.4 γ of 5HTA, *D* = platelets, *E* = platelets + 0.7 γ of 5HTA, *F* = platelets + 1.4 γ of 5HTA.

spectrophotofluorometer described here is that one can readily obtain two spectra for each analysis instead of a single reading on a galvanometer. Thus, one obtains direct evidence of specificity for individual analyses.

DISCUSSION

Recent interest in the biochemistry and physiology of 5HTA makes it desirable to have available sensitive and specific analytical procedures for this compound and related metabolites. The three methods used to measure 5HTA can also be applied to other 5-hydroxyindole derivatives by appropriate extraction procedures. Thus, it has been possible to develop a specific procedure for measuring 5-hydroxyindoleacetic acid in urine based on the nitrosonaphthol reaction following its extraction into ether.[3] At

[3] Udenfriend, S., Weissbach, H., and Titus, E., to be published.

present the colorimetric and spectrophotometric methods are best suited to metabolic studies which involve the assay of 5HTA in tissues to which it or its precursors have been added in relatively large amount. They have been used successfully in many enzymatic studies in this laboratory. Certain tissues, such as rabbit and beef platelets and rabbit stomach and intestine, contain sufficient 5HTA to permit assay by the colorimetric procedure. However, unless large quantities of human blood are used, the colorimetric procedure falls far short of the required sensitivity. The fluorometric procedure on the other hand is sufficiently sensitive to measure the 5HTA in several ml. of blood.

It should be noted that 5HTA is activated with ultraviolet light and emits ultraviolet-fluorescent light. Since fluorescence in the ultraviolet is a rather general phenomenon (6), it should be applicable to many other analytical problems when suitable and simple instruments become available.

SUMMARY

A procedure is described for extracting 5-hydroxytryptamine (serotonin) from biological tissues for analytical purposes. Three methods are described for assaying 5-hydroxytryptamine in these extracts. The first is by measurement of its ultraviolet absorption; the second is by colorimetric estimation of the chromophore produced by reaction with 1-nitroso-2-naphthol; and the third is by measurement of its fluorescence in solution.

The last method utilizes a spectrophotofluorometer which can activate and measure emitted fluorescence continuously from 250 to 650 mμ. 5HTA and other 5-hydroxyindoles are activated maximally at 295 mμ and emit fluorescent light which shows a maximum at 330 mμ.

The fluorescence procedure has been applied to the measurement of 5HTA in platelets. As little as 0.05 γ of 5HTA can be measured.

BIBLIOGRAPHY

1. Clark, C. T., Weissbach, H., and Udenfriend, S., *J. Biol. Chem.*, **210,** 139 (1954).
2. Gerngross, O., Voss, K., and Herfelt, T., *Ber. chem. Ges.*, **66,** 435 (1933).
3. Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, **196,** 227 (1952).
4. Udenfriend, S., and Weissbach, H., *Federation Proc.*, **13,** 412 (1954).
5. Zucker, M. B., and Rapport, M. M., *Federation Proc.*, **13,** 170 (1954).
6. Bowman, R. L., and Udenfriend, S., *Science*, in press.
7. Dillard, G. H. L., Brecher, G., and Cronkite, E. P., *Proc. Soc. Exp. Biol. and Med.*, **78,** 853 (1951).

THE CATABOLISM OF C^{14}-LABELED β-ALANINE IN THE INTACT RAT

By ALEXANDER PIHL* AND PER FRITZSON

(From Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway)

(Received for publication, December 3, 1954)

The recent work of Fink *et al.* (1, 2) indicating that β-alanine is an intermediate in pyrimidine catabolism has greatly increased the interest in β-alanine metabolism in animal tissues. Its rôle in synthetic reactions has, in part, lately been clarified in studies demonstrating that β-alanine is utilized in the formation of carnosine (3, 4) and anserine (4). However, only limited information is available concerning its oxidative metabolism. It has been found both *in vivo* (5) and *in vitro* (6, 7) that β-alanine is rapidly deaminated, and in certain tissues incubated with β-alanine the formation of ketone bodies has been demonstrated (8, 9). However, the initial reaction product has not been identified and the fate of the carbon chain remains obscure.

The purpose of this work is to study the rate and the mode of oxidation of β-alanine in animal tissues. We have found that β-alanine is rapidly and extensively oxidized in the intact rat. Evidence is presented to show that after deamination the carboxyl carbon is liberated by decarboxylation, while the rest of the carbon chain is metabolized by way of acetic acid.

EXPERIMENTAL

Preparation of Labeled Compounds—C^{14}-β-Alanine labeled separately in each of the three positions was prepared as described previously (10).

Respiration Experiments—The animals were albino rats, weighing 160 to 180 gm. Equal numbers of male and female rats were used. They had previously been fed on a stock diet and were not fasted before use.

The rats were injected intraperitoneally with 1.0 to 1.4 mg. of β-alanine singly labeled with C^{14} in the 1, 2, or 3 position. The β-alanine was administered as the free amino acid in approximately 3 ml. of 0.9 per cent sodium chloride. Immediately after injection each rat was placed in a glass metabolism chamber (11). The expired CO_2 was collected in CO_2-free NaOH, from which it was precipitated as $BaCO_3$ (12), and the radioactivity was determined. During the first 2 hours the CO_2 was collected every 15 minutes, during the following 3 hours, every 30 minutes.

Feeding Experiments—Three female rats, weighing 160 to 176 gm., were

* Fellow of the Norwegian Cancer Society.

fed β-alanine labeled in the 1, 2, and 3 positions, respectively, together with *p*-aminobenzoic acid. The animals were kept in metabolism cages and given a diet consisting of bread and meat extract made into a paste. The β-alanine (0.5 mmole per 100 gm. of rat tissue per day) was administered by stomach tube on the 1st day and mixed with the food on the 2 following days. The *p*-aminobenzoic acid (100 mg. per 100 gm. of rat tissue per day) was mixed with the food. The rations were so adjusted that every day all the food given was consumed. After 3 days feeding, the animals were sacrificed and the livers removed.

Acetyl-*p*-aminobenzoic acid was isolated from the urine and purified as described by Bloch and Rittenberg (13). Cholesterol and total fatty acids were isolated from the livers after complete alkaline hydrolysis. Cholesterol was precipitated from the unsaponifiable fraction as the digitonide. The total fatty acids were extracted from the acidified hydrolysate according to conventional procedures.

Determination of Radioactivity—The labeled organic compounds were converted to barium carbonate after wet combustion (12) and the samples counted with a thin window Geiger-Müller counter. All samples were counted for a sufficient length of time to give a standard error of less than 3 per cent. The data were corrected when necessary for mass absorption to give the number of counts per minute of infinitely thick samples (4.75 sq. cm.).

The isotope concentration of the acetyl group of *p*-aminobenzoic acid was obtained by multiplying the acetyl-*p*-aminobenzoic acid value by 9/2. Similarly, the specific activity of cholesterol was obtained by multiplying the digitonide value by 82/27.

For comparison of data from different experiments the "concentration coefficient" (*CC*) is used. This quantity is the relative isotope concentration (*RIC*) which would have been found if 1 mmole of the test substance had been given per 100 gm. of rat tissue per day (14).

$$CC = \frac{RIC}{Q + (1 - Q)RIC}$$

where *RIC* = (isotope concentration of the isolated compound)/(isotope concentration of the administered compound) and *Q* is the quantity of the compound fed in millimoles per 100 gm. of rat tissue per day (14).

Results

Oxidation of β-Alanine—Fig. 1 shows the C^{14} concentration of the respiratory CO_2 after intraperitoneal injection of labeled β-alanine. It is apparent that β-alanine is metabolized at a strikingly rapid rate. After injection of *carboxyl-labeled* β-alanine the isotope concentration of the CO_2 reached

high values within the first 15 minutes, and the peak concentration was reached after approximately 25 minutes. In 2 hours as much as 89 per cent of the injected C^{14} was excreted.

The curves further demonstrate that the 3 atoms of the carbon chain were excreted at distinctly different rates, reflecting different metabolic pathways. The *carboxyl carbon atom* was excreted at its maximal rate about 30 minutes after injection, while after 2 to 3 hours the C^{14} content of the expired air was very low. On the other hand, *carbon atom 2* was

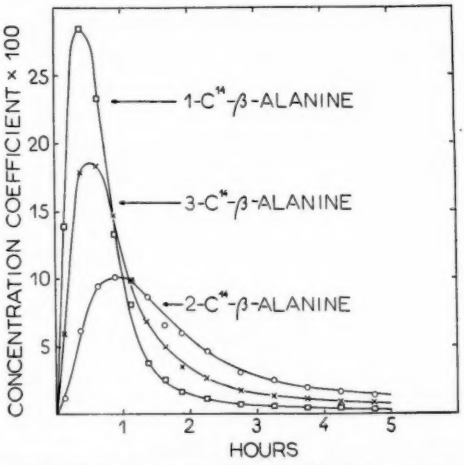


FIG. 1. C^{14} concentration of respiratory CO_2 following intraperitoneal injection of β-alanine labeled separately in each of the 3 different carbon atoms. Isotope concentration is expressed as the "concentration coefficient;" *i.e.*, the relative isotope concentration per millimole fed per day per 100 gm. of rat tissue. Each curve represents the data from two separate experiments.

excreted far more slowly. The maximal rate of excretion occurred about 60 minutes after injection, and the curve then declined slowly. After 2 hours substantial amounts of radioactivity were still present in the CO_2. The excretion of *carbon atom 3* occurred at an intermediate rate.

The cumulative excretion of C^{14} in 5 hours was 93 per cent,[1] 60 per cent, and 77 per cent of the dose after administration of 1-, 2-, and 3-labeled β-alanine respectively.

Conversion to Acetate—In order to test the possible conversion of β-alanine to acetic acid, the incorporation of the 3 different carbon atoms of

[1] Martignoni and Winnick have recently reported (4) that approximately 90 per cent of the C^{14} was recovered in expired CO_2 4 hours after the administration of 1-C^{14}-β-alanine to rats. However, no details were given.

β-alanine into the acetyl group of acetyl-*p*-aminobenzoic acid and into the liver fatty acids and cholesterol was tested.

Comparison of the concentration coefficients of the acetyl groups (Table I) demonstrates that carbon atoms 2 and 3 of β-alanine were utilized efficiently and to approximately the same extent as sources of acetyl carbon, whereas the carboxyl carbon was utilized only to an insignificant extent. Clearly, this finding provides strong evidence that the α- and β-carbon atoms of β-alanine are metabolized by way of acetic acid. The further finding that the α- and β-carbon atoms were both efficiently incorporated into fatty acids and cholesterol, while in comparison the incorporation of carboxyl carbon was almost negligible, provides additional evidence in

TABLE I

Incorporation of C^{14} from β-Alanine into Acetyl Groups of Acetyl-p-aminobenzoic Acid of Urine and into Liver Fatty Acids and Cholesterol

Compound fed, per 100 gm. per day	C^{14} concentration	C^{14} concentration of					
		Acetyl carbon*		Fatty acids		Cholesterol	
			CC†		CC†		CC†
	mmole	*c.p.m.*‡	*c.p.m.*‡	*per cent*	*c.p.m.*‡	*per cent*	*c.p.m.*‡	*per cent*
1-C^{14}-β-Alanine	0.49	77,000	30	0.08	5	0.01	10	0.03
2-C^{14}-β-Alanine	0.50	87,900	758	1.71	100	0.23	290	0.66
3-C^{14}-β-Alanine	0.50	34,300	390	2.25	144	0.84	255	1.48

* Acetyl group of acetyl-*p*-aminobenzoic acid of urine.

† The relative isotope concentration per millimole fed per day per 100 gm. of rat tissue.

‡ Counts per minute of $BaC^{14}O_3$.

support of this view. The hypothesis that β-alanine is metabolized by way of acetic acid is consistent with the previous findings of Heinsen (8, 9) that ketone bodies were formed when β-alanine was incubated with slices from liver or heart muscle.

If carbon atoms 2 and 3 of β-alanine are converted to acetate, they should be incorporated in the ratio of 1:1 into fatty acids and to approximately the same extent into cholesterol (15).[2] It is evident from Table I that marked deviations from these ratios were actually observed. Thus in the experiment with 3-C^{14}-β-alanine the concentration coefficients for fatty acids and cholesterol were both several times higher (3 and 2.2 times) than in the experiment with 2-C^{14}-β-alanine. However, in recent experiments in which labeled acetate as such was administered intraperi-

[2] The methyl and carboxyl carbon atoms of acetic acid are incorporated into cholesterol in the ratio of 15:12 (15).

toneally to rats, individual differences of the same magnitude were observed with regard to the incorporation of C^{14} into fatty acids and cholesterol (16, 17). Consequently, the deviations from the theoretical ratios observed in the present two experiments do not constitute evidence against the view that the 2 and 3 carbons of β-alanine are utilized for lipide synthesis in the same ratio as acetate.

DISCUSSION

Two facts seem to be clearly established from the present work; *i.e.*, that β-alanine is rapidly and extensively oxidized and that part of its carbon chain is converted to acetic acid.

Although the formation of β-alanine from dihydrouracil has been demonstrated in liver slices (1, 2), its physiological significance in the breakdown of the pyrimidines is not yet clear. If β-alanine lies in the normal path of pyrimidine catabolism, the amounts formed per day may not be inconsiderable. It is a fact, however, that normally only small amounts of β-alanine are excreted in the urine (18, 19). This might be explained by the rapid oxidation demonstrated in the present work.

As to the mechanism of the conversion of β-alanine to acetic acid, the evidence here presented is only suggestive. It is likely that the first step in the oxidative metabolism of β-alanine involves loss of the amino group. Enzyme systems capable of effecting transamination of β-alanine with α-ketoglutaric acid have been demonstrated in liver and brain tissues (7). In a study of β-alanine metabolism by ground rat kidney cortex (6) added β-alanine disappeared, with the formation of CO_2 and NH_3. Ethylamine and hydroxyethylamine did not appear as intermediate products and did not undergo deamination, indicating that decarboxylation did not occur prior to deamination.

The initial product of the deamination has not been identified, but it appears reasonable to assume that formylacetic acid is formed, in analogy with the formation of succinaldehyde from γ-aminobutyric acid (20). Formylacetic acid is unstable and has not been isolated. Attempts to isolate the free acid resulted in its decomposition into carbon dioxide and acetaldehyde (21). If β-alanine is metabolized by way of formylacetic acid, as indicated, the carboxyl carbon will necessarily be excreted at the fastest rate in the respiratory CO_2 (as it is liberated directly by decarboxylation), while the remaining carbon atoms will be excreted more slowly after oxidation of acetaldehyde by way of acetic acid. It is a further corollary that carbon atom 2 of β-alanine should be excreted more slowly than carbon atom 3, since the methyl carbon of acetate is more slowly oxidized than the carboxyl carbon (22, 11). Clearly, the data presented here on the relative rates of excretion of the 3 different carbon atoms are consistent with the above hypothesis.

The possibility that β-alanine carbon is incorporated into acetate by routes other than the proposed decarboxylation of formylacetic acid should also be considered. Conversion by way of propionic or pyruvic acid can be excluded as significant pathways, since these acids, in contrast to β-alanine, are glycogenic (23). In the formation of acetate from propionate and to a lesser extent from pyruvate (24) randomization occurs between carbon atoms 2 and 3, indicating that equilibration with symmetrical compounds, probably C_4 acids, takes place prior to acetyl formation. If a 3-carbon intermediate derived from β-alanine were similarly equilibrated with symmetrical C_4 acids of the Krebs cycle, the label from β-alanine might appear in the acetyl groups by several indirect routes (25), all of which would involve a redistribution of the tracer. The fact that in the present experiments distinctly different rates of C^{14} elimination were found when 2- and 3-labeled β-alanine were given indicates that no extensive randomization had taken place in the conversion of β-alanine to acetate. The data therefore seem to favor the view that β-alanine is metabolized via a direct route by way of formylacetic acid-acetaldehyde-acetic acid.

SUMMARY

The metabolism of β-alanine has been studied in the intact rat by measuring the incorporation of its carbon atoms into respiratory CO_2, into the acetyl group of excreted acetyl-*p*-aminobenzoic acid, and into cholesterol and fatty acids of the liver.

β-Alanine is rapidly and extensively metabolized. After intraperitoneal injection more than 90 per cent of the radioactivity of injected 1-C^{14}-β-alanine was recovered in the expired CO_2 in 5 hours. Carbon atom 3 and particularly carbon atom 2 were excreted at distinctly slower rates, 77 and 60 per cent of the dose, respectively, being excreted in 5 hours.

Carbon atoms 2 and 3 were efficiently incorporated into excreted acetyl-*p*-aminobenzoic acid and into fatty acids and cholesterol, whereas the incorporation of the carboxyl carbon into these compounds was negligible.

The pathway suggested involves deamination of β-alanine to formylacetic acid, with subsequent decarboxylation to carbon dioxide and acetaldehyde, which is further oxidized to acetic acid.

The technical assistance of Miss Unni Evenrud is gratefully acknowledged.

BIBLIOGRAPHY

1. Fink, R. M., Fink, K., and Henderson, R. B., *J. Biol. Chem.*, **201**, 349 (1953).
2. Fink, K., and McGaughey, C., *Federation Proc.*, **13**, 207 (1954).
3. Williams, H. M., and Krehl, W. A., *J. Biol. Chem.*, **196**, 443 (1952).
4. Martignoni, P., and Winnick, T., *J. Biol. Chem.*, **208**, 251 (1954).

5. Graff, J., and Hoberman, H. D., *J. Biol. Chem.*, **186,** 369 (1950).
6. Sorvachev, K. F., *Biokhimiya*, **18,** 696 (1953); *Chem. Abstr.*, **48,** 7658 (1954).
7. Roberts, E., and Bregoff, H. M., *J. Biol. Chem.*, **201,** 393 (1953).
8. Heinsen, H. A., *Ergeb. inn. Med. u. Kinderh.*, **54,** 672 (1938).
9. Heinsen, H. A., *Z. exp. Med.*, **114,** 755 (1945).
10. Fritzson, P., and Eldjarn, L., *Scand. J. Clin. and Lab. Invest.*, **4,** 375 (1952).
11. Weinhouse, S., and Friedmann, B., *J. Biol. Chem.*, **191,** 707 (1951).
12. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F., Isotopic carbon, New York (1949).
13. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159,** 45 (1945).
14. Anker, H. S., *J. Biol. Chem.*, **176,** 1337 (1948).
15. Little, H. N., and Bloch, K., *J. Biol. Chem.*, **183,** 33 (1950).
16. Hutchens, T. T., Van Bruggen, J. T., Cockburn, R. M., and West, E. S., *J. Biol. Chem.*, **208,** 115 (1954).
17. Van Bruggen, J. T., Yamada, P., Hutchens, T. T., and West, E. S., *J. Biol. Chem.*, **209,** 635 (1954).
18. Stein, W. H., *J. Biol. Chem.*, **201,** 45 (1953).
19. Crokaert, R., Contribution à l'étude de la β-alanine et de ses composés dans les milieux biologiques, Brussels (1953).
20. Bessman, S. P., Rossen, J., and Layne, E. C., *J. Biol. Chem.*, **201,** 385 (1953).
21. Tschitschibabin, A. E., *J. prakt. Chem.*, **73,** 326 (1906).
22. Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K., and Hastings, A. B., *J. Biol. Chem.*, **177,** 295 (1949).
23. Schofield, F. A., and Lewis, H. B., *J. Biol. Chem.*, **169,** 373 (1947).
24. Shreeve, W. W., *J. Biol. Chem.*, **195,** 1 (1952).
25. Topper, Y. J., and Stetten, D., Jr., *J. Biol. Chem.*, **209,** 63 (1954).

SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF 5-KETO-D-GLUCONIC ACID

By D. PERLMAN

(From The Squibb Institute for Medical Research, New Brunswick, New Jersey)

(Received for publication, December 14, 1954)

Methods for the determination of 5-keto-D-gluconic acid in the presence of the reducing sugars found in fermentation media have been proposed by Stubbs *et al.* (1) and by Militzer (2). In the former, the 5-keto-D-gluconic acid is separated from the fermented medium by precipitation as the calcium salt, dissolved in an oxalic acid solution, and determined by reduction of the Shaffer-Hartmann alkaline copper reagent and volumetric determination of the quantity of the cuprous oxide precipitate. In the method described by Militzer the rate of reaction of 5-keto-D-gluconic acid with Benedict's reagent at 25° is visually observed, and the quantity of the 5-keto-D-gluconic acid present is determined by comparison with a standard curve. Both of these methods require large quantities of 5-keto-D-gluconic acid (about 50 mg.) and are time-consuming. While Militzer's method has inherently a high degree of specificity, compared with that of Stubbs, the variation in analyses found between analysts, and between determinations on a single sample by one analyst, may be large. Plotting the standard curve on a sheet of semilog paper eased calculation of results, but even with this modification the precision was found to be low, especially when colored solutions were analyzed.

We have found that spectrophotometric measurement of the reduction of Nelson's (3) arsenomolybdate reagent at 50° may be used to determine the quantity of 5-keto-D-gluconic acid present in solutions. The reaction is specific under these conditions, and as little as 5 γ of the acid can be determined rapidly.

Procedure

An aliquot of an aqueous solution containing between 5 and 80 γ of 5-keto-D-gluconic acid is added to a test-tube. Sufficient distilled water is added to give a total volume of 1 ml., and 5 ml. of the arsenomolybdate reagent of Nelson (3) diluted 1:3 with distilled water are added. The mixture is placed for a period of 2 hours in a 50° water bath, and the color intensity then determined in an Evelyn photoelectric colorimeter equipped with a 660 mμ filter. A blank is run on the reagents. A series of tubes containing known quantities of 5-keto-D-gluconic acid[1] is included in each

[1] A sample of potassium salt of 5-keto-D-gluconic acid was used as the reference

XUM

set of analyses. The color intensity follows Beer's law over the range of 5 to 80 γ per tube. By comparison with the curve obtained with the known amounts, the quantity of 5-keto-D-gluconic acid in each aliquot of unknown concentration can be readily calculated.

DISCUSSION

As can be seen in Fig. 1, the rate of reaction of the 5-keto-D-gluconic acid with the arsenomolybdate reagent is a function of temperature. Complete reaction occurs within 15 minutes at 100°, but under these conditions the reagent is reduced by any reducing sugar and by many other compounds. When the reaction temperature was lowered to 50°, and a 2 hour

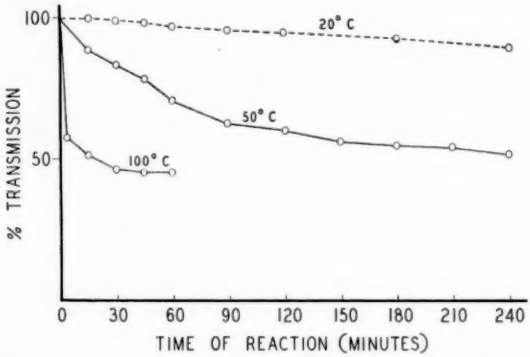


FIG. 1. Effect of reaction temperature on reduction of arsenomolybdate reagent by 5-keto-D-gluconic acid (10 γ per tube).

heating period was used, greater specificity in the reaction is found. Under these conditions there is no significant reduction of the reagent by glucose, mannose, sorbose, galactose, maltose, gluconic acid, glucuronic acid, or galacturonic acid, even when these substances are present at levels of 800 γ per tube. At temperatures above 70° aldohexoses cause significant reduction of the reagent. If a reaction period shorter than 2 hours is used, the sensitivity of the method is reduced as well as is the precision because a significant reduction of the reagent occurs while the measurements of color are being made. After the 2 hour reaction period this factor is insignificant.

Under the conditions outlined above, fructose and sucrose will react with the arsenomolybdate reagent. Fructose was found to have about 8 per cent and sucrose about 3 per cent the reducing power of 5-keto-D-

standard in all of the experiments. It was found to contain 99 per cent potassium 5-keto-D-gluconate by elementary analysis (anhydrous basis).

gluconic acid. These sugars and 5-keto-D-gluconic acid may be identified qualitatively by the filter paper chromatographic method described by Koepsell *et al.* (4). If sucrose or fructose is present in significant amounts, *e.g.* 5 times the concentration of 5-keto-D-gluconic acid or more, it can be separated quantitatively from the acid by passing the sample over the

TABLE I

Analysis of Solutions for 5-Keto-D-gluconic Acid

Solution	Composition of solution	5-Keto-D-gluconic acid added	5-Keto-D-gluconic acid found	
			Proposed method	Militzer's method
		mg. per ml.	*mg. per ml.*	*mg. per ml.*
A	Glucose, 50 gm.; corn steep-liquor, 3 gm.; $MgSO_4 \cdot 7H_2O$, 0.25 gm.; KH_2PO_4, 0.6 gm.; NH_4NO_3, 4 gm.; pH adjusted to 7.0 with NaOH; distilled water to 1 liter	0	<0.08, 0.07	
		1	0.95, 0.97	
		10	9.3, 10	11, 12
B	Glucose, 50 gm.; Na_2HPO_4, 0.5 gm.; corn steep-liquor, 1.3 ml.; $MgSO_4 \cdot 7H_2O$, 0.16 gm.; $NaNO_3$, 3 gm.; $CaCO_3$, 57 gm.; H_3BO_3, 9.6 gm.; distilled water to 1 liter	0	<0.09, <0.08	
		1	1.04, 1.0	
		10	9.5, 10	11, 13
C	Sucrose, 50 gm.; corn steep-liquor, 3 gm.; $MgSO_4 \cdot 7H_2O$, 0.25 gm.; KH_2PO_4, 0.6 gm.; NH_4NO_3, 4 gm.; pH adjusted to 7.0 with NaOH; distilled water to 1 liter	0	1.88, 1.75	
		0	<0.08,* <0.08	
		1	1.1,* 1.1	
		10	9.3,* 9.7	11, 13
D	Glucose, 100 gm.; NH_4NO_3, 4 gm.; KH_2PO_4, 1.6 gm.; corn steep-liquor, 3 gm.; $CaCO_3$, 28 gm.; distilled water to 1 liter	0	<0.08	
		10	9.7	
E	D fermented with *Acetobacter suboxydans* for 6 days; treated as described by Khesghi *et al.* (6)	0	59, 58	64, 70
		10	68, 69	75, 80

* After treatment with resin IRA-400, as described in the text.

anion exchange resin IRA-400[2] prepared for use as described by Kunin and McGarvey (5). The acid is retained on the resin while the sugars are not. The acid is then eluted by washing the resin with 5 times its volume of 0.08 N H_2SO_4. The eluate is then diluted to a known volume and the 5-keto-D-gluconic acid content of the solution determined as above. Analyses of several types of solutions are summarized in Table I. These results show

[2] A product of The Resinous Products Division, Rohm and Haas Company, Philadelphia, Pennsylvania.

that in practically all cases the ingredients present in the microbiological media did not reduce the reagent, that satisfactory recovery of 5-keto-D-gluconic acid from various fermentation samples was obtained, and that the method has a high degree of precision. The analytical values obtained agreed with those obtained by Militzer's method.

The above procedure may also be used for the determination of 2-keto-D-gluconic acid. Under the above conditions this acid has approximately 16 per cent of the reducing power of 5-keto-D-gluconic acid. The method has been found to be as accurate as, and less laborious and more sensitive than, that proposed by Stubbs *et al.* (1) for determination of 2-keto-D-gluconic acid.

We are indebted to Dr. F. H. Stodola and Mr. G. E. N. Nelson for samples of potassium 5-keto-D-gluconate.

SUMMARY

A micromethod for the determination of 5-keto-D-gluconic acid based on the reduction of the arsenomolybdate reagent at 50° has been studied. By use of this method it has been possible to determine 5 γ of 5-keto-D-gluconic acid in fermentation samples.

BIBLIOGRAPHY

1. Stubbs, J. J., Lockwood, L. B., Roe, E. T., Tabenkin, B., and Ward, G. E., *Ind. and Eng. Chem.*, **32,** 1626 (1940).
2. Militzer, W. E., *J. Biol. Chem.*, **154,** 325 (1944).
3. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
4. Koepsell, H. J., Stodola, F. H., and Sharpe, E. S., *J. Am. Chem. Soc.*, **74,** 5142 (1952).
5. Kunin, R., and McGarvey, F. X., *Ind. and Eng. Chem.*, **41,** 1265 (1949).
6. Khesghi, S., Roberts, R. R., and Bucek, W., *Appl. Microbiol.*, **2,** 183 (1954).

ERYTHROCYTE PRESERVATION

IV. IN VITRO REVERSIBILITY OF THE STORAGE LESION*

By BEVERLY WESCOTT GABRIO, MARION HENNESSEY, JOAN THOMASSON, AND CLEMENT A. FINCH

(From the Department of Medicine, University of Washington School of Medicine, and the King County Central Blood Bank, Seattle, Washington)

(Received for publication, December 20, 1954)

Previous investigations (1–3) have shown that the biochemical lesion occurring during the storage of blood in acid-citrate-dextrose at 4° is primarily an intrinsic metabolic failure of the erythrocyte. Alterations in extraerythrocytic factors such as plasma, leucocytes, reticulocytes, hemolysates, citrate, and gas phase were ineffective in alleviating or accelerating the degenerative process in the red cell during storage. The storage lesion was found also to be unrelated to senescence of the red cell *in vivo*.

The biochemical lesion of storage was rapidly reversed after the introduction of stored cells into the active circulation of a normal animal as measured by a regeneration of cellular organic phosphates, especially ATP.[1] A similar reversal was found to occur also in the transfused erythrocytes of man. These restorative changes were interpreted to indicate an increased metabolic potential of the cell and were paralleled by other physiological alterations of the cell. This *in vivo* reversibility of the changes occurring in the stored cell appeared to be an all or none phenomenon. If the storage damage had not reached a critical point whereby the cells were lost from circulation within 24 hours after transfusion, the phosphate partition and other attendant systems of the remaining cells returned to normal within a few hours.

The present study is concerned with the reversibility of the biochemical lesion of storage *in vitro*, with special reference to the use of the nucleoside, adenosine.

* This investigation was supported in part by research grants from the United States Atomic Energy Commission (contract AT(45-1)-343), the Department of the Army, Office of the Surgeon General (contracts DA-49-007-MD-508 and DA-49-007-MD-512), and Initiative 171, State of Washington, funds for research in Biology and Medicine. The authors wish to express their sincere appreciation to Dr. F. M. Huennekens for his interest and advice in many phases of the work presented in this series.

[1] ATP = adenosine triphosphate; ADP = adenosine diphosphate; TPN = triphosphopyridine nucleotide.

Materials and Methods

Acid-citrate-dextrose preservative[2] was used routinely for the storage of human and rabbit blood at 4°. The final concentration of the preservative components in the blood was approximately 0.264 per cent sodium citrate, 0.096 per cent citric acid, and 0.294 per cent glucose.

Phosphate partitions on suspensions of erythrocytes in 0.9 per cent NaCl were performed as described previously (1).

Adenosine concentration was calculated from the absorption at 258 mμ as measured with a Beckman model DU spectrophotometer; a millimolar extinction coefficient of 14.1 was used (4). For the calculation of adenosine concentration in plasma, the difference in absorption at 258 mμ between adenosine plus plasma and a plasma control was employed.

Glucose was determined colorimetrically (5) on a blood filtrate prepared according to the method of Somogyi (6).

The subcellular components of rabbit liver were prepared according to the procedure of Lee and Williams (7), except that the nuclear, mitochondrial, and microsomal + residual fractions were obtained by successive centrifugations of the homogenate at 600 × *g* for 10 minutes and at 29,000 × *g* for 30 minutes respectively.

EXPERIMENTAL

Effect of Subcellular Components of Liver on Storage Lesion—To test the action of liver particulates *in vitro*, stored blood was incubated at 37° for 1 to 2 hours with the liver fractions, and phosphate partitions were carried out on the red cells at the end of this time (Table I). Phosphate analyses of the particulate fractions alone indicated that, in the amounts used, there was negligible contribution of the particulates *per se* to the red cell phosphate values if any cross-contamination existed. Furthermore, in preparation of red cells for analysis, it was possible to remove most of the particulate fraction as well as white cells which sedimented above the erythrocytes. From the results of a typical experiment shown in Table I, it is apparent that the mitochondrial fraction provided some stimulus for the resynthesis of organic phosphates. The phosphorus was derived partially from the cellular inorganic phosphorus fraction, which had increased during storage, and from inorganic phosphorus withdrawn from the plasma medium. Apparently a diffusible substance from the mitochondria was the active agent. Although the regeneration of organic phosphates of the stored cells was significant, the reversibility was not comparable to that obtained *in vivo* as demonstrated previously. The cellular difficultly hydrolyzable phosphate fraction was not increased after treatment with liver particulates.

[2] National Institutes of Health, Formula B.

Substances such as succinate, α-ketoglutarate, fumarate, and malate, dinitrophenol, coenzyme A, various amino acid mixtures, and nicotinamide, when added to blood either alone or in combination with the liver fractions, neither enhanced nor inhibited the changes observed with incubated stored cells alone or those observed with only liver particulate additions.

Effect of Adenosine Triphosphate Derivatives on Storage Lesion—Compounds such as adenine, ribose, inorganic orthophosphate, adenosine, and adenylic acid were incubated 1 hour with stored blood, and the phosphate partitions of the red cells were determined. In Table II are recorded the results of a typical experiment in which 20 μmoles of each substance were used. It is apparent that supplemental adenosine provided the greatest

TABLE I

Effect of Liver Particulates on Stored Erythrocytes

8 ml. aliquots of rabbit blood (27 days stored) were incubated for 1 hour at 37° with 0.5 ml. of liver particulate, adjusted so that the concentration of the particulate was equivalent to that present in a 10 per cent homogenate.

Sample	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Control stored blood	1207	131	155	704	2197
Blood + homogenate	1204	155	130	740	2229
" + nuclear fraction	1203	153	139	715	2210
" + mitochondrial fraction	1176	173	134	869	2352
" + microsomal + residual fraction	1205	153	118	758	2234

impetus to the resynthesis of red cell organic phosphates and that a noteworthy reconstitution was obtained in all fractions. Although both muscle and yeast adenylic acids caused some hemolysis of the red cells, they proved to be relatively stimulatory. The apparent uptake of the latter two compounds was a somewhat unexpected finding owing to the known limited permeability of phosphorylated compounds. Ribose-5-phosphate, ATP, and ADP were found to yield negative results in whole cell systems. There was no adenosine synthesis from the addition of adenine and ribose, as expected, since ribose-1-phosphate is required for this nucleoside synthetic mechanism.

The variation in the amounts of total cellular phosphorus in the experimental samples (Table II) can be accounted for by differences in retention of inorganic phosphorus after incubation. After exposure of stored blood to 37°, there is a diffusion of some of the accumulated inorganic phosphorus

to the extracellular environment (Table III). Treated stored blood retains varying amounts of this phosphorus for the resynthesis of organic phosphates and often withdraws phosphorus from the plasma, depending upon the effectiveness of the added substance. In other cases, in which there is no rejuvenation of treated cells, the membrane permeability may be changed by the agent, thus allowing more or less inorganic phosphorus diffusion.

In all of the experiments described, whole cell systems were used, and the uptake of the adenine-containing compounds tested except ATP and ADP was about the same; *i.e.*, 50 to 70 per cent in 1 hour at 37°.

TABLE II

Effect of ATP Derivatives on Stored Erythrocytes

9 ml. aliquots of human blood (stored 27 days) were incubated 1 hour at 37° with 20 μmoles of the specific compound indicated. The hematocrit of the cell systems was 30.

Sample	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Control	686	155	52	113	1006
Adenine	621	150	50	130	951
Ribose	653	134	39	163	989
Adenine + ribose	617	170	10	161	958
" + " + inorganic orthophosphate	780	182	32	78	1072
Adenosine	269	301	236	323	1129
" + inorganic orthophosphate	460	303	211	322	1296
Muscle adenylic acid	662	184	68	200	1114

Characterization of Adenosine Effect—A similar reversal was produced by adenosine in stored rabbit and human erythrocytes, and the following results are considered equally applicable to both species.

(*a*) "*Absorption*" *of Adenosine*—Inasmuch as the uptake of adenosine by the red cell could be absorption of the compound into the cell or adsorption onto the membrane, the phenomenon will be referred to hereafter as "absorption." The absorption of adenosine presented to the cells in optimal concentration to produce the red cell effect (2500 μmoles per 100 ml. of red blood cells) was tested at 37° with human blood (stored 30 days) over a period of 1.5 hours. The per cent absorption was as follows: 15 minutes 35 per cent, 30 minutes 44 per cent, 1 hour 61 per cent, and 1.5 hours 71 per cent. These values were reproducible in all experiments with stored blood. Accordingly, most of the reversibility studies have been

carried out over a period of 1 hour at 37° and about 60 per cent absorption of adenosine was obtained.

(*b*) *Concentration of Adenosine*—The effect of varying concentrations of adenosine on human red cells stored 25 days is shown in Table III. The optimal concentration of adenosine to produce maximal reconstitution of cellular organic phosphates was approximately 2500 μmoles of adenosine per 100 ml. of red cells. At this concentration of added substance, the total phosphorylated compounds were regenerated to a level which was comparable to that present in fresh blood. The absorption of adenosine by

TABLE III

Concentration of Adenosine and Rejuvenation of Stored Erythrocytes

11 ml. aliquots of human blood (stored 25 days) were incubated 1 hour at 37° with increasing amounts of adenosine.

Adenosine added per 100 ml. red blood cells	Absorption of adenosine	μmoles phosphorus per 100 ml. red blood cells				
		Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
μmoles	*per cent*					
Fresh		165	230	181	922	1498
Stored 25 days						
Non-incubated control		945	169	81	79	1274
Incubated control		680	185	71	67	1003
125	100	613	262	77	132	1084
375	74	440	325	151	256	1172
625	64	299	362	243	355	1259
1250	68	151	407	311	384	1253
2500	62	98	436	361	461	1356
7500	31	99	438	375	461	1373

the erythrocytes varied somewhat with the amount added to the blood. However, within a concentration range of 625 to 2500 μmoles per 100 ml of red blood cells, about two-thirds of the added nucleoside was absorbed.

An examination of the efficiency of adenosine indicates that over a wide range of adenosine concentration a resynthesis of organic phosphates occurs (Fig. 1). A linear relationship appears to obtain, and approximately 1 μmole of organic phosphate is resynthesized for 1 μmole of adenosine absorbed. At higher concentrations of the nucleoside the ratio of micromoles of organic phosphate resynthesized to micromoles of adenosine absorbed declines; this is probably a reflection of more absorption than utilization of the adenosine molecule.

A Michaelis constant, K_m, of 3.6×10^{-3} mole per liter was obtained from data in which 125 to 7500 μmoles of adenosine per 100 ml. of red blood cells

were incubated for 1 hour at 37°. In these computations, v = the organic phosphate resynthesized, expressed as μmoles of organic P per 100 ml. of red blood cells per hour, and s = the concentration of adenosine absorbed,

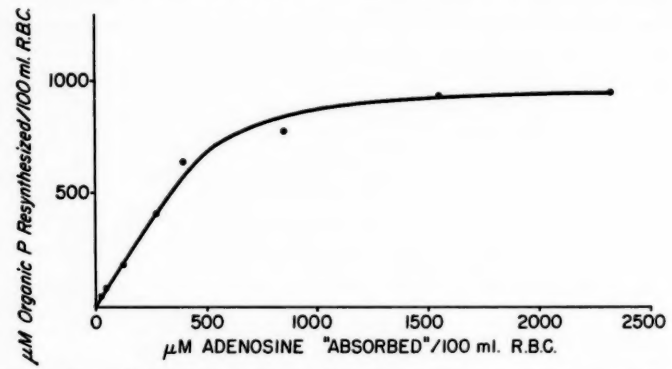


FIG. 1. The relationship between adenosine absorption and the resynthesis of erythrocyte organic phosphates. Stored blood was incubated for 1 hour at 37° with varying concentrations of adenosine.

TABLE IV

Effect of Temperature on Rejuvenation of Stored Red Cells with Adenosine

11 ml. aliquots of human blood (stored 28 days) were exposed to adenosine in the concentration of 2400 μmoles per 100 ml. of red blood cells at 2°, 23°, and 37° for 1 hour.

	Absorption of adenosine	μmoles phosphorus per 100 ml. red blood cells					
		Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total	Increase in organic P
	per cent						
2°. Control		808	163	87	155	1213	
2°. Adenosine	24	654	230	124	220	1228	169
23°. Control		754	199	80	127	1160	
23°. Adenosine	50	337	272	258	395	1262	519
37°. Control		559	206	47	177	989	
37°. Adenosine	69	88	376	376	473	1313	795

expressed as μmoles per 100 ml. of red blood cells per hour, and the conventional plot of $1/v$ and $1/s$ was employed.

(*c*) *Effect of Temperature—In vitro* reversibility with adenosine occurred at 2° as well as 37° (Table IV). Although the increase in organic phosphates resynthesized in stored blood exposed to adenosine at 2° for

1 hour was only 169 μmoles per 100 ml. of red cells compared with 795 μmoles in blood incubated at 37°, the amount of adenosine penetrating the cell at the lower temperature was approximately one-third that found at 37°. The calculated activation energy for this process, which is a composite of the resynthesis of cellular organic phosphates and the diffusion of adenosine through the cell barrier, was 6540 calories per mole per degree over the temperature range of 2–37°. It was demonstrated in other experiments that full restoration did occur at 2° over a longer period of time.

(*d*) *Adenosine Effect As Related to Duration of Storage*—The effect of adenosine on blood stored for 4 weeks has been illustrated in Tables II to IV. In Table V the effect of adenosine on fresh blood is illustrated. It will be observed that even those cells containing a normal phosphate composition

TABLE V

Effect of Adenosine on Fresh and Stored Red Cells

10 ml. aliquots of human blood were incubated with adenosine (1500 μmoles per 100 ml. of red blood cells) for 45 minutes at 37°.

	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Fresh. Control	245	304	161	776	1486
" Adenosine	39	375	230	1002	1646
Stored 12 days. Control	592	269	124	332	1317
" 12 " Adenosine	111	449	309	734	1603

increased in the organic phosphate fractions. In the same blood stored for 12 days and treated with adenosine there was a net resynthesis of organic phosphate which was almost twice that produced by stimulation of fresh cells. Apparently adenosine is more effective on the degenerate system. However, the absolute content of organic phosphate in the treated 12 day cells was less than that of the treated fresh cells, but more than was present in the control fresh cells. It is of interest that those erythrocytes not hemolyzed after 120 days of storage in acid-citrate-dextrose at 4° will also show some improvement in their phosphate partition after incubation with adenosine.

(*e*) *Glucose Concentration Related to Adenosine Effect*—In all of the reversibility experiments described previously, whole blood containing acid-citrate-dextrose was employed. Thus a factor to be considered is whether or not the reversal phenomenon will occur in red cell suspensions devoid of the substances (especially glucose) which are present in the plasma-acid-

citrate-dextrose medium. Optimal levels of adenosine were incubated for 1 hour at 37° with suspensions of stored erythrocytes in 0.9 per cent NaCl containing from 14 to 2600 μmoles of glucose per 100 ml. of red blood cells. The absorption of adenosine in each case was 65 to 69 per cent. The results indicated that the presence or absence of glucose did not affect the resynthesis of organic phosphate esters during adenosine treatment for 1 hour.

(*f*) *Aerobic Utilization of Glucose by Rejuvenated Cells*—The previous experiments have described the reversal effect of adenosine in terms of the

TABLE VI

Aerobic Utilization of Glucose by Adenosine-Treated Stored Erythrocytes

Aliquots of human blood (stored 28 days) were incubated 1 hour at 37° with 2100 μmoles of adenosine per 100 ml. of red blood cells. Cell suspensions of the incubated samples were prepared, after washing once, in Krebs-Ringer-phosphate solution. Hemolysates were prepared on twice washed cells by freezing and thawing three times. The stroma fraction was removed by centrifugation. O_2 uptake was measured for 2 hours in the conventional Warburg apparatus at 37°; O_2 gas phase.

		μl. O_2 per hr. per ml. red blood cells
Cell suspension.	Control	36
" "	Adenosine	121
Hemolysate.	Control	371
"	Adenosine	389

The *cell suspension system* contained, in the main compartment, 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of 1.8 per cent glucose, 1.00 ml. of cell suspension, 1.69 ml. of 0.1 M phosphate buffer, pH 7.5; in the center well, 0.20 ml. of 5 N NaOH. The *hemolysate system* contained, in the main compartment, 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of glucose-6-phosphate (100 μmoles per ml.), 0.30 ml. of TPN (1 mg. per ml.), 0.50 ml. of glucose-6-phosphate dehydrogenase (4 mg. per ml.), 0.30 ml. of nicotinamide (100 μmoles per ml.), 0.10 ml. of 1 M ethyl alcohol, 0.05 ml. of catalase, 1.00 ml. of hemolysate, 0.44 ml. of 0.1 M phosphate buffer, pH 7.5; in the center well, 0.20 ml. of 5 N NaOH. Huennekens, F. M., Liu, L., Myers, H., and Gabrio, B. W., unpublished observations.

resynthesis of organic phosphorylated compounds of red cells. It was of interest to determine whether adenosine-treated stored cells were able to utilize glucose at an increased rate. Two systems were employed to measure glucose utilization: red cell suspensions and hemolysates; the former employed the substrate glucose, the latter glucose-6-phosphate. The following electron transport mechanism obtains in both systems (8).

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-phosphate} + \text{TPN} \rightarrow \text{6-phosphogluconate} + \text{TPNH} \quad (2)$$

$$\text{TPNH} + O_2 \rightarrow \text{TPN} + H_2O_2 \quad (3)$$

$$H_2O_2 + \text{EtOH} \rightarrow H_2O + CH_3CHO \quad (4)$$

Reactions 1 to 4 are catalyzed, respectively, by hexokinase, glucose-6-phosphate dehydrogenase, methemoglobin reductase with methylene blue as cofactor, and catalase.

The results presented in Table VI indicate that the utilization of glucose by the rejuvenated blood in the cell suspension experiment was about 4-fold greater than that of the control, while there was no difference in oxygen uptake in the hemolysate systems. It would appear that the limiting step in the stored blood is the one shown in Reaction 1. The specific limitation is apparently a greatly diminished ATP level, since it has been shown[3] that hexokinase activity is unchanged throughout storage. Adenosine stimulation results in the resynthesis of ATP, thus allowing the phosphorylation of glucose to proceed at a faster rate.

DISCUSSION

Prominent among the changes occurring during storage of blood are a decreased utilization of glucose and the depletion of organic phosphate content of the erythrocyte. The demonstration of a rapid return to normal of the phosphate partition of stored blood, once injected into the blood stream, led to a search for the mechanism of this reversal. In rabbits hepatectomized by the two-stage procedure (9), little or no reversal of the phosphate partition was observed over 1 hour after hepatectomy.[4] Therefore, liver fractions were prepared in the hope of isolating the substance or causative system responsible for the reversal *in vivo*. While definite increases in red cell organic phosphate were observed with the use of the mitochondrial fraction, the magnitude of these changes was not comparable to those obtained with reversal *in vivo*.

In a more direct attempt to reestablish glucose utilization and intracellular ATP, substances chemically related to ATP were added. Adenosine was found to produce repletion of ATP and other organic phosphates and to accelerate the aerobic utilization of glucose to even a greater extent than had been observed previously *in vivo*. Ribose, phosphorylated ribose, orthophosphate ion, and adenine were without effect. It appeared that the riboside was needed to produce both adequate penetration of the cell and to enter thereafter into the metabolic scheme of the cell.

Adenosine was similarly effective in the stored erythrocytes of both rabbit and man. This is consistent with the fact that the storage lesion is an intrinsic cellular defect and that the abnormalities occur at the same rate in both rabbit and human blood *in vitro*. Upon incubation at 37° for 1 hour with optimal concentrations of adenosine, about two-thirds of the nucleoside was absorbed by the erythrocytes, and the regeneration oc-

[3] Private communication, Dr. E. G. Krebs.

[4] Gabrio, B. W., Maguire, R., Donohue, D., and Finch, C. A., unpublished observations.

curred independently of glucose content. However, this should not be interpreted to mean that a portion of the adenosine molecule may replace glucose completely, although this possibility does exist, at least for short term experiments. While the reversal is nearly complete in 1 hour at 37°, several hours at 2° are required to produce the same effect. It is significant that the reversal will occur at the standard storage temperature used in the preservation of blood. However, it was found that glucose was essential for adenosine to function in its maximal capacity in maintaining the erythrocyte during long term storage at 4°. This aspect will be dealt with further in a subsequent communication.

It is of interest that adenosine can increase the organic phosphate content of fresh cells and can produce also some effect on non-viable cells stored for as long as 120 days in acid-citrate-dextrose. The latter observation would indicate that the metabolic pathway through which adenosine exerts its effect is a stable one and not easily destroyed.

The ultimate test of treated stored cells is the ability of these cells to survive after transfusion. It has been found that posttransfusion viability of adenosine-treated erythrocytes is considerably prolonged (10).

The mode of action of adenosine at the molecular level in the repair of the biochemical lesion of stored erythrocytes is unknown at the present time. However, inasmuch as there is an almost 1:1 stoichiometry involving adenosine and organic phosphate resynthesized, the reaction appears to be a chemical rather than a physical effect. Assuming then a chemical reaction, and with the knowledge that ribose and adenine alone are ineffective, adenosine apparently carries ribose into the cell where the nucleoside is subsequently cleaved to its purine base and ribose-1-phosphate. The existence of a nucleoside phosphorylase in this reaction is implicit in the work of Dische (11) and has been demonstrated in this laboratory with inosine as a substrate (12). Ribose-1-phosphate would then participate in the resynthesis of the organic phosphate esters, possibly through the aerobic shunt pathway. The K_m of 3.6×10^{-3} mole per liter and activation energy of 6540 calories per mole per degree both suggest that enzymatic reactions are involved in the stimulation by adenosine. Studies are currently in progress to investigate this problem.

The authors wish to thank Mrs. Alice Rupen and Miss Frances Vokracek for technical assistance in the early phases of the work involving liver particulates.

SUMMARY

The *in vitro* reversibility of the biochemical lesion of the stored erythrocyte has been demonstrated to occur to a slight extent with subcellular

components of the liver, to a moderate extent with some ATP derivatives, and completely with adenosine. It would appear that the repair of the storage lesion with adenosine involves enzymatic reactions in which the ratio of micromoles of organic phosphorus resynthesized to micromoles of adenosine absorbed is unity. A K_m of 3.6×10^{-3} mole per liter and activation energy of 6540 calories per mole per degree were calculated for this system.

BIBLIOGRAPHY

1. Gabrio, B. W., and Finch, C. A., *J. Clin. Invest.*, **33,** 242 (1954).
2. Gabrio, B. W., Stevens, A. R., Jr., and Finch, C. A., *J. Clin. Invest.*, **33,** 247 (1954).
3. Gabrio, B. W., Stevens, A. R., Jr., and Finch, C. A., *J. Clin. Invest.*, **33,** 252 (1954).
4. Schmitz, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **209,** 41 (1954).
5. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
6. Somogyi, M., *J. Biol. Chem.*, **160,** 69 (1945).
7. Lee, N. D., and Williams, R. H., *Endocrinology*, **54,** 5 (1954).
8. Huennekens, F. M., and Gabrio, B. W., *Federation Proc.*, **13,** 232 (1954).
9. Drury, D. R., *J. Exp. Med.*, **49,** 759 (1929).
10. Gabrio, B. W., Donohue, D. M., and Finch, C. A., *J. Clin. Invest.*, in press.
11. Dische, Z., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 171 (1951).
12. Gabrio, B. W., and Huennekens, F. M., *Federation Proc.*, **14,** 217 (1955).

THE RÔLE OF RIBOFLAVIN AND VITAMIN B_6 IN TRYPTOPHAN METABOLISM*

By L. M. HENDERSON, RUTH E. KOSKI,† and F. D'ANGELI‡

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois)

(Received for publication, December 27, 1954)

From the pattern of urinary products excreted following the administration of tryptophan and some of its metabolites by vitamin-deficient rats, certain suggestions regarding the rôle of riboflavin and vitamin B_6 have been made. The most characteristic changes resulting from these deficiency states are decreased formation of pyridine derivatives (*N'*-methylnicotinamide (1, 2), quinolinic acid (2), and pyridine nucleotides (3, 4)) and increased excretion of xanthurenic acid (5, 6). Impairment of the action of kynureninase, the enzyme which catalyzes the splitting of kynurenine and hydroxykynurenine as well as other α-amino-γ-keto acids (7) to alanine and the corresponding anthranilic acid, has been offered as the explanation for the disturbed metabolism noted in vitamin B_6 deficiency.

The involvement of riboflavin in the hydroxylation of kynurenine was suggested by increased kynurenic acid (8) and decreased pyridine derivatives in the urine of deficient rats given tryptophan or kynurenine (2). Further support for this idea was provided by the observation that riboflavin-deficient rats excreted more anthranilic acid and its derivatives in response to tryptophan (7, 9). This suggested rôle of riboflavin has been questioned (7) on the basis of the finding that more xanthurenic acid (XA) is excreted by riboflavin-deficient rats given tryptophan than by normal animals. Our earlier observations (2) involving the excretion patterns following the administration of tryptophan, kynurenine, and 3-hydroxyanthranilic acid have been extended to include 3-hydroxykynurenine. The quinolinic acid (QA) excretion findings support the postulated rôle of riboflavin in the hydroxylation of kynurenine. While riboflavin deficiency increased the xanthurenic acid response to tryptophan, it decreased slightly the response to kynurenine. An explanation for the effect of riboflavin deficiency on xanthurenic acid excretion is offered.

* Supported by a grant in aid from E. I. du Pont de Nemours and Company, Inc., to the Department of Chemistry and Chemical Engineering, University of Illinois, and by a grant from the United States Public Health Service (No. 2674).

† Some of these data have been taken from a thesis submitted by Ruth E. Koski in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois.

‡ Present address, Istituto di Chimica Farmaceutica, Università di Padova, Padova, Italy.

XUM

EXPERIMENTAL

Diets and Animals—The diets were identical to those used in the earlier studies (2). Male rats from the local colony were used. They were fed the experimental diets when they reached 50 gm. in weight for vitamin B_6 deficiency and 60 gm. for riboflavin deficiency. The food intake of the non-deficient control animals and of the vitamin B_6-deficient rats was restricted to that of the riboflavin-deficient animals. When the growth of the riboflavin-deficient rats had ceased, the vitamin B_6 deficiency was accentuated by feeding the same deficient diet to which 1 mg. per cent of 4-deoxypyridoxine had been added. Within 3 to 5 days the vitamin B_6 deficiency had become so severe that weight loss had occurred and symptoms began to appear. At this time the animals received orally a supplement containing the vitamins, excluding nicotinic acid, present in 10 gm. of their respective diets.[1] They were then placed in individual metabolism cages and 24 hour control urine samples collected. At this time L-tryptophan or L-kynurenine sulfate in 0.9 per cent sodium chloride solution or DL-kynurenine, 3-hydroxy-DL-kynurenine sulfate, or 3-hydroxyanthranilic acid in a saline suspension at pH 6.0 to 7.0 was administered intraperitoneally in four equal doses 3 hours apart. The experimental collection of urine was made during the 24 hour period following the first injection. The urine samples were stored under toluene at 5° until analyzed.

Nicotinic acid and quinolinic acid were estimated microbiologically (10), and xanthurenic acid by the method of Rosen *et al.* (11).

Test Compounds—L-Tryptophan, a commercial product, melted at 245–246° with decomposition and had a specific rotation of −30.0° in water at 25°.

L-Kynurenine was isolated from rabbit urine as the sulfate by the method of Kallio and Berg (12), following the administration by stomach tube of 3 gm. of L-tryptophan per day. The sulfate had a specific rotation of +11.7° in water at 25° and melted at 160–165° with decomposition and gas evolution. It gave only one fluorescent spot at R_F = 0.47 when chromatographed in the solvent system of Mason and Berg (13). The absorption spectrum and analysis gave the expected results. In the later studies L-kynurenine sulfate was prepared by the method of Warnell and Berg (14) by ozonolysis of acetyl-L-tryptophan prepared by the method of du Vign-

[1] In some of the experiments preliminary to those reported here, control animals receiving a diet containing all the vitamins except nicotinic acid failed to convert tryptophan to quinolinate and nicotinate to the usual extent. Additional studies failed to demonstrate conclusively any impairment of the conversion of tryptophan to nicotinate by niacin-deficient animals. Undoubtedly the niacin deficiency is relieved rather quickly by the test dose of tryptophan.

eaud and Sealock (15). The product had a specific rotation at 20° of +12.03° in water, and melted with decomposition at 163–164°.

The DL-kynurenine sulfate was the same sample previously used (2). Oxygen consumption data,[2] obtained during its oxidation by resting cells of a strain of *Pseudomonas fluorescens* which oxidizes only the L isomer, suggest that the product is the racemic mixture with a slight preponderance of the L isomer. This is in good agreement with the analytical results and the presence of only slight optical activity.

3-Hydroxy-DL-kynurenine was prepared by the method of Musajo, Spada, and Casini (16) from 2-nitro-3-methoxybenzoic acid prepared by nitration of 3-methoxybenzoic acid with concentrated nitric acid (17) or with acetyl nitrate (18). Better yields were realized when the condensation of phenacyl bromide and acetamidomalonic ester was effected by heating at 60° for 3 to 4 hours. 2-Nitro-3-methoxybenzoylalanine was obtained in 38 per cent yield from the phenacyl bromide. The yield of hydroxy-DL-kynurenine from 2-nitro-3-methoxybenzoic acid was 11.6 per cent. The product decomposed on heating on the Kofler micro block at 200–217°.

$C_{10}H_{12}O_4N_2$.	Calculated,	C 53.57, H 5.40, N 12.50
	Found,	" 53.82, " 5.68, " 12.35

The absorption spectrum of the product coincided with that of a sample kindly supplied by Dr. Musajo and with that published by Dalgliesh (19). Chromatography on paper in the solvent system used by Mason and Berg (13) gave two green fluorescent spots which appeared identical under ultraviolet light, at R_F 0.37 and 0.31. The separation of the enantiomorphic forms of kynurenine with this solvent system has been reported. Dalgliesh (19) reported resolution of hydroxy-DL-kynurenine in a different solvent system. Chromatograms of urine from normal rats, following administration of this racemic product, showed the presence of the $R_F = 0.31$ spot but none of the other isomer, whereas, on chromatograms of urines from vitamin B_6-deficient rats following L-tryptophan or L-kynurenine, only the compound with $R_F = 0.37$ was detected. From these results and those of Dalgliesh it is suggested that hydroxy-L-kynurenine is the compound at $R_F = 0.37$, while the D isomer appears at $R_F = 0.31$. Efforts are being made to identify positively the separated products.

From preliminary experiments a daily dosage of 0.25 mmole of hydroxy-DL-kynurenine was selected. Larger quantities caused a toxic reaction, characterized by a short period of pain and semiconsciousness following each dose, with no increase in the proportion of the dose converted to pyridine derivatives.

[2] Kindly measured by Dr. R. Y. Stanier, University of California, Berkeley, California.

XUM

RESULTS AND DISCUSSION

The mean nicotinic acid excretion during the control period was 23 γ for control animals, 15 γ for riboflavin-deficient, and 16 γ for vitamin B_6-deficient animals. The mean quinolinic acid excretions were 49, 31, and 38 γ, respectively.

Table I shows the increase in nicotinic acid excretion over the control period for each compound tested. In each case vitamin B_6 deficiency reduced the niacin excretion. Riboflavin deficiency had little effect on the conversion of any of the compounds, except tryptophan, to urinary niacin. The increase in quinolinic acid excretion over control period values was approximately 10 to 25 times as great as the increase in nicotinic acid, except after 3-hydroxyanthranilic acid, when the increase in quinolinate was 60

TABLE I

*Effect of Riboflavin and Pyridoxine Deficiencies on Excretion of Nicotinic Acid**

Compound injected, 0.25 mmole	No. of rats per group	Nicotinic acid above control period		
		Control	Riboflavin-deficient	Pyridoxine-deficient
		γ	γ	γ
L-Tryptophan	11	150	30	16
L-Kynurenine	6	20	16	6
DL-Kynurenine	4	9	7	4
3-Hydroxy-DL-kynurenine	10	14	9	4
3-Hydroxyanthranilate	11	82	79	33

* Expressed as the mean increase over the control period excretion.

to 100 times as great. It is felt that the greater percentage conversion and the more consistent results with quinolinic acid make this compound a more reliable indication of the animal's capacity to convert tryptophan and its metabolites to pyridine derivatives.

A summary of the results of three separate experiments involving 126 24 hour test periods is shown in Table II. The results are presented as the total 24 hour excretions and as percentage of the test compound administered. In agreement with earlier observations, riboflavin deficiency caused less formation of urinary quinolinate from tryptophan, L-kynurenine, and DL-kynurenine, but not from 3-hydroxyanthranilate. Likewise, the excretion of quinolinate in response to 3-hydroxy-DL-kynurenine was not significantly reduced in this deficiency.

While these results indicated clearly that riboflavin deficiency was affecting the conversion of L- and DL-kynurenine, but not 3-hydroxy-DL-kynurenine to quinolinate, the effect on kynurenine conversion was not as large as

in the case of vitamin B_6 deficiency. In an effort to magnify the difference in conversion of kynurenine to quinolinate caused by this vitamin deficiency, attempts were made to accentuate the deficiency. Animals were fed the deficient diet described above until they exhibited severe hair loss, dermatitis, and other signs of ariboflavinosis. In spite of these efforts their responses to kynurenine and hydroxykynurenine did not differ significantly from those shown in Table II.

In two experiments galactoflavin[3] (20) was incorporated into the diet in a further attempt to intensify the deficiency. In the first of these experiments, 2 mg. per cent of galactoflavin were included in the diet of deficient and control animals after 45 days on experiment. The riboflavin

TABLE II

Effect of Riboflavin and Pyridoxine Deficiencies on Quinolinic Acid Excretion

Compound injected, 0.25 mmole	No. of rats per group	Conversion to quinolinic acid in		
		Control	Riboflavin-deficient	Pyridoxine-deficient
		γ	γ	γ
L-Tryptophan	11	4411 ± 642* (10.6 ± 1.5%)*	514 ± 66 (1.23 ± 0.16%)	131 ± 34 (0.31 ± 0.083%)
L-Kynurenine	6	625 ± 97 (1.5 ± 0.23%)	326 ± 44 (0.78 ± 0.11%)	86 ± 13 (0.206 ± 0.032%)
DL-Kynurenine	4	190 ± 9 (0.45 ± 0.02%)	154 ± 8 (0.37 ± 0.019%)	53 ± 25 (0.127 ± 0.062%)
3-Hydroxy-DL-kynurenine	10	250 ± 9 (0.59 ± 0.02%)	233 ± 13 (0.56 ± 0.03%)	37 ± 5 (0.088 ± 0.011%)
3-Hydroxyanthranilic acid	11	4875 ± 558 (11.7 ± 1.34%)	5423 ± 579 (12.99 ± 1.39%)	3071 ± 494 (7.36 ± 1.18%)

* Standard error of the mean.

content of the diet of the control group was increased to 1.2 mg. per 100 gm. The test compounds were administered 15 days later. In the second experiment the galactoflavin was incorporated into the diet at a level of 10 mg. per cent beginning on the 12th day, and experiments were conducted on the 50th and 60th days of the deficiency period.

A summary of the results of these studies is presented in Table III. The conversion of kynurenine to quinolinate was reduced somewhat more than in the previous experiments. In agreement with the data in Table II, riboflavin deficiency had no significant effect on the conversion of hydroxykynurenine to quinolinate.

From the results obtained thus far, it was evident that the deficiency of riboflavin exerted a less profound effect on the metabolism of kynurenine

[3] Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

than did vitamin B_6. This could mean that the riboflavin-containing coenzymes are not so readily reduced to a critical level for this metabolic process or that the riboflavin effect is indirect. As expected from previous work, the vitamin B_6-deficient animals (Table II) excreted very much less quinolinate in response to tryptophan and kynurenine, but not to 3-hydroxyanthranilic acid. In agreement with the postulated rôle of vitamin B_6 in the removal of the side chain of hydroxykynurenine, this

TABLE III

Effect of Galactoflavin-Induced Riboflavin Deficiency on Quinolinate Response to Tryptophan and Its Metabolites

Experiment No.	Compound injected, 0.25 mmole	No. of rats per group	Increase in quinolinate excretion over control period	
			Control group	Riboflavin-deficient
			γ per 24 hrs.	*γ per 24 hrs.*
1	L-Tryptophan	2	3028 (2370–3680)	659 (414–905)
	DL-Kynurenine	2	1100 (800–1400)	662 (468–856)
	Hydroxy-DL-kynurenine	2	153 (110–196)	412 (369–455)
	3-Hydroxyanthranilate	2	6350 (2360–10,350)	6140 (5300–7000)
2	L-Kynurenine	3	599 (476–780)	199 (105–298)
	Hydroxy-DL-kynurenine	4	217 (174–238)	151 (111–240)

TABLE IV

Effect of Vitamin Deficiencies on Xanthurenic Acid Excretion

Compound injected, 0.25 mmole	No. of rats	Controls		No. of rats	Riboflavin-deficient		No. of rats	Pyridoxine-deficient	
		γ	*per cent*		*γ*	*per cent*		*γ*	*per cent*
L-Tryptophan.....	5	269	0.53	5	3870	7.6	2	15,650	31.5
Kynurenine.....	11	4,960	9.8	11	3640	7.2	2	6,660	13.0
3-Hydroxykynurenine.....	11	12,500	24.5	11	9200	18.1	2	9,730	19.1

deficiency reduced the conversion of this compound from 0.59 to 0.088 per cent.

In view of the report (7) that xanthurenic acid excretion following tryptophan administration was elevated in riboflavin-deficient rats, representative urine samples from the experiments described above were analyzed for this metabolite. A summary of the values is presented in Table IV. These findings agree well with those of Musajo *et al.* (21) for normal animals and with those of Charconnet-Harding *et al.* (7) for normal and riboflavin-deficient rats. While the data presented in Tables II and III are consistent with the view that hydroxylation of kynurenine becomes impaired in ariboflavinosis, the increased xanthurenic acid formation (0.53

to 7.6 per cent) from tryptophan seems contrary to this view. An examination of the level of excretory products following kynurenine administration, however, indicates that lack of riboflavin reduces XA excretion slightly. It is interesting that in control animals tryptophan is converted to the extent of 10 per cent to QA and 0.5 per cent to XA, while kynurenine is converted to the extent of 9.8 per cent to XA and about 1 per cent to QA. In the riboflavin-deficient animals, about the same amount of both metabolites was excreted whether tryptophan or kynurenine was given, and the excretion pattern approximated that of control animals given kynurenine. This means that in this deficiency the metabolism of tryptophan resembles that of kynurenine in a normal or deficient animal. This could be interpreted as indicating an accumulation of kynurenine when tryptophan is administered to riboflavin-deficient rats. Such a pattern might be expected if the utilization of kynurenine is impaired.

Paper chromatograms of urine of riboflavin-deficient animals which received tryptophan revealed the presence of compounds which would be expected if kynurenine hydroxylation were impaired. These included anthranilic and kynurenic acids and two less well established compounds. On the basis of R_F values, negative ninhydrin test, appearance under ultraviolet light, and ultraviolet absorption spectrum, one component was tentatively identified as the compound described by Dalgliesh (19) and thought to be N^{α}-acetylkynurenine. The other appears to be a derivative of anthranilic acid. Kynurenine administration to riboflavin-deficient rats gave similar papergram patterns and hydroxykynurenine showed no anthranilic acid or derivative, and no N^{α}-acetylkynurenine.

The vitamin B_6 deficiency was characterized by the excretion of only trace amounts of quinolinate following the administration of hydroxykynurenine and its precursors, presumably because the removal of the side chain to form 3-hydroxyanthranilic acid is effectively blocked. The relatively large total excretion of products which can arise from hydroxykynurenine, particularly when tryptophan was administered, points to the partial blocking of alternative pathways such as the formation of anthranilic acid from kynurenine when vitamin B_6 is not provided in adequate amounts. It should be pointed out that the 19 to 25 per cent conversion of hydroxykynurenine to XA is based on experiments with the racemate. It seems likely that the D isomer is not metabolized in this manner, since, in all cases, after injection of hydroxy-DL-kynurenine animals excreted large amounts of a fluorescent compound which appeared to be unchanged hydroxy-D-kynurenine.

It is evident that further information regarding the site of action of B vitamins in this metabolic sequence will have to await studies *in vitro*. Attempts to hydroxylate kynurenine *in vitro* with liver and kidney preparations have met with little success. On the other hand rat liver slices

and homogenates convert approximately 25 per cent of hydroxy-DL-kynurenine to quinolinate during 3 hours of incubation. This system provides a suitable method of studying the removal of the side chain, since the product 3-hydroxyanthranilate can be estimated fluorometrically (22) or enzymatically by determining quinolinate.

SUMMARY

1. The effect of administering tryptophan and its metabolites, including 3-hydroxykynurenine, on the excretion of niacin, quinolinic acid, and xanthurenic acid in normal and vitamin-deficient rats has been determined.

2. The excretion of products containing the pyridine nucleus in response to hydroxykynurenine was reduced in vitamin B_6-deficient rats, but not in the riboflavin-deficient animals.

3. The xanthurenic acid excretion in response to tryptophan was increased by the riboflavin deficiency, but the xanthurenic acid response to kynurenine and 3-hydroxykynurenine was reduced slightly.

4. The results offer some support for the idea that hydroxylation of kynurenine is impaired by lack of riboflavin.

BIBLIOGRAPHY

1. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **168,** 555 (1947).
2. Henderson, L. M., Weinstock, I. M., and Ramasarma, G. B., *J. Biol. Chem.*, **189,** 19 (1951).
3. Ling, C.-T., Hegsted, D. M., and Stare, F. J., *J. Biol. Chem.*, **174,** 803 (1948).
4. Kring, J. P., Ebisuzaki, K., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **195,** 591 (1952).
5. Lepkovsky, S., and Nielsen, E., *J. Biol. Chem.*, **144,** 135 (1942).
6. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, **157,** 551 (1945); **159,** 173 (1945).
7. Charconnet-Harding, F., Dalgliesh, C. E., and Neuberger, A., *Biochem. J.*, **53,** 513 (1953).
8. Porter, C. C., Clark, I., and Silber, R. H., *Arch. Biochem.*, **18,** 339 (1948).
9. Mason, M., *J. Biol. Chem.*, **201,** 513 (1953).
10. Henderson, L. M., *J. Biol. Chem.*, **181,** 677 (1949).
11. Rosen, F., Lowy, R. S., and Sprince, H., *Proc. Soc. Exp. Biol. and Med.*, **77,** 399 (1951).
12. Kallio, R. E., and Berg, C. P., *J. Biol. Chem.*, **181,** 333 (1949).
13. Mason, M., and Berg, C. P., *J. Biol. Chem.*, **188,** 783 (1951).
14. Warnell, J. L., and Berg, C. P., *J. Am. Chem. Soc.*, **76,** 1708 (1954).
15. du Vigneaud, V., and Sealock, R. R., *J. Biol. Chem.*, **96,** 511 (1932).
16. Musajo, L., Spada, A., and Casini, E., *Gazz. chim. ital.*, **80,** 171 (1950).
17. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **70,** 1847 (1948).
18. D'Angeli, F., Koski, R. E., and Henderson, L. M., *J. Biol. Chem.*, **214,** 781 (1955).
19. Dalgliesh, C. E., *Biochem. J.*, **52,** 3 (1952).
20. Emerson, G. H., Wurtz, E., and Johnson, O. H., *J. Biol. Chem.*, **160,** 165 (1945).
21. Musajo, L., Chiancone, F. M., and Coppini, D., *Science*, **113,** 125 (1951).
22. Long, C. L., Hill, H. N., Weinstock, I. M., and Henderson, L. M., *J. Biol. Chem.*, **211,** 405 (1954).

XUM

EFFICIENCY OF PHOSPHORYLATION IN SELECTED OXIDATIONS BY MITOCHONDRIA FROM NORMAL AND THYROTOXIC RAT LIVERS*

By GLADYS FELDOTT MALEY and HENRY A. LARDY

(From the Department of Biochemistry and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin)

(Received for publication, December 17, 1954)

In this laboratory, two lines of experimentation have been employed to test the hypothesis that the effects of the thyroid hormone are the result of uncoupling oxidative phosphorylation reactions (1, 2). In the first case enzyme preparations from thyrotoxic rat livers were less efficient in coupling phosphorylation and oxidation than were similar preparations from normal rats (2, 3). Studies *in vitro* have shown that, under specified conditions, both thyroxine and triiodothyronine depressed P:O ratios[1] of normal kidney mitochondrial preparations (2, 4). Both lines of evidence indicated that thyroid hormones influence phosphate-transferring reactions. However, neither demonstrated the specific site of action of the hormone in the electron transport sequence, since only the over-all efficiency of oxidative phosphorylation was measured.

In the hope that a study of specific oxidations by thyrotoxic tissues would clarify the rôle of the hormone in phosphate-transferring reactions, a comparison of the phosphorylations associated with a number of one-step oxidations by normal and thyrotoxic rat liver mitochondria has been made.

EXPERIMENTAL

150 to 200 gm. rats (Holtzman strain) maintained on a natural ration were used throughout the study as normal controls. Unless otherwise designated, thyrotoxicity was produced by feeding the natural ration supplemented with 2 per cent desiccated thyroid powder (Wilson). For comparative purposes, some rats were rendered thyrotoxic by intraperitoneal injections of thyroxine or triiodothyronine. Animals fed the desiccated thyroid material were maintained on this regimen for at least 7 to 10 days and not longer than 24 days prior to sacrifice. This regimen produced an acute thyrotoxicity to which the animal did not become refractory.

* Supported in part by grants from the Office of Naval Research and the National Vitamin Foundation.

[1] The following abbreviations are used: ATP, adenosine triphosphate; P:O, micromoles of inorganic phosphate uptake per microatoms of O uptake; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide; Q_{O_2} (N), c.mm. of O_2 consumed per hour per mg. of mitochondrial nitrogen.

Liver mitochondria were isolated by the method of Schneider (5) from a normal and a thyrotoxic rat at the same time. Each ml. of the final suspensions represented a gm. of fresh liver. The reaction mixtures will be described with the experiments. Hexokinase was purified from yeast to Stage 3A by the method of Berger *et al.* (6). ATP was obtained from the Pabst Laboratories as the disodium salt.

The techniques employed for determining efficiency of oxidative phosphorylation have been described previously (7, 8). All experiments were performed in duplicate and the average values are recorded. The quantity of mitochondria used per flask varied from 1.5 to 2.2 mg. of N. The duration of the experiments was from 15 to 25 minutes, but in most cases was 20 minutes. For purposes of comparing the respiration in the phosphorylation experiments, the oxygen consumption has been calculated to a uniform time and nitrogen basis.

TABLE I

Comparison of Over-All Efficiency of Phosphorylations Accompanying Oxidation of Glutamate to Succinate by Liver Mitochondria from Normal and Thyrotoxic Rats

Treatment	No. of animals	O uptake*	P:O
		μatoms	
Control	16	7.9	3.2 ± 0.025†
Thyroxine-injected	9	9.5	2.2 ± 0.36
Triiodothyronine-injected	7	8.9	2.4 ± 0.39
Desiccated thyroid in ration	7	9.2	1.7 ± 0.7

The reaction vessels contained 6 μmoles of ATP, 50 μmoles of phosphate buffer, pH 7.4, 15 μmoles of $MgSO_4$, 0.03 μmole of cytochrome *c*, 60 μmoles of glutamate, 10 μmoles of malonate, 50 μmoles of glucose, hexokinase, mitochondria from 0.5 gm. of fresh liver, and isotonic sucrose to a final volume of 3.0 ml. Equilibration period, 8 minutes; 0.2 ml. of 5 N KOH in the center well; gas phase, air; temperature, 30°. Thyroxine and triiodothyronine were injected intraperitoneally at a dose of 2 mg. per day for 4 days.

* Per 20 minutes and calculated on the basis of 2 mg. of mitochondrial N.

† Standard deviation = $\sqrt{\Sigma d^2/(n-1)}$.

Results

The data in Table I summarize the results of a comparison of the over-all efficiency of the phosphorylations accompanying the oxidation of glutamate to succinate by normal and thyrotoxic rat liver mitochondria. In all cases, regardless of the method employed in producing thyrotoxicity, the P:O ratio was lower in mitochondria from thyrotoxic animals. In sixteen experiments the normal P:O ratio averaged 3.2, while with mitochondria from animals injected with thyroxine or triiodothyronine, or fed desiccated thyroid, the P:O ratios were 2.2, 2.4, and 1.7, respectively.

Comparable results were obtained with α-ketoglutarate as substrate (Table II). The P:O ratio in the control experiments averaged 3.6, whereas an average value of 2.7 was obtained in seven experiments with mitochondria from livers of rats fed desiccated thyroid. Similar depressions of phosphorylation efficiency accompanying ketoglutarate oxidation were found in mitochondria from a limited number of rats injected with thyroxine or triiodothyronine at the dosage level described in Table I.

It should be noted that, in the presence of phosphate acceptor, mitochondria from thyrotoxic rats consume oxygen at a faster rate than does

TABLE II

Comparison of Over-All Efficiency of Phosphorylations Associated with Oxidation of α-Ketoglutarate to Succinate by Liver Mitochondria from Normal and Thyrotoxic Rats

Normal		Thyrotoxic	
O uptake*	P:O	O uptake*	P:O
μatoms		*μatoms*	
7.7	3.5	10.3	3.1
8.7	3.2	7.2	2.0
6.0	3.9	12.2	3.1
8.0	3.6	10.3	3.0
6.2	3.6	9.0	3.2
		9.2	2.1
		9.5	2.5
Average...7.2	3.6	9.7	2.7

Reaction mixtures were the same as for Table I, except that 20 μmoles of α-ketoglutarate were substituted for glutamate as substrate, and the malonate level was increased to 30 μmoles.

* Per 20 minutes and calculated on the basis of 2 mg. of mitochondrial N.

an equivalent amount of mitochondrial nitrogen from normal rats. These data, and those of Aebi and Abelin (9), who studied succinate oxidation by mitochondria, differ from those of Hoch and Lipmann (10), who reported that mitochondria from thyrotoxic rats have a lower respiration than that from the controls.

It has been reported by Lipmann and coworkers (10, 11) that the addition of a phosphate acceptor system to mitochondria from thyrotoxic rat liver does not increase the oxygen consumption as it does with normal liver mitochondria (11, 12). However, preliminary experiments with mitochondria from rats made thyrotoxic as described above indicated that the phosphate acceptor effect was of lesser magnitude than usual, but was not absent (13). Further experiments were therefore performed and the aver-

age results of nine experiments are presented in Table III (Series H). In the absence of phosphate acceptor, mitochondria from the thyrotoxic rats

TABLE III

Influence of Phosphate Acceptor on Respiration of Liver Mitochondria from Normal, Hyperthyroid, and Thyroidectomized Rats

Series	Q_{O_2} (N)			
	Normal rats		Thyrotoxic rats	
	No acceptor	+ ~P acceptor	No acceptor	+ ~P acceptor
H,* average of 9 experiments	64	516 (8×)†	162	574 (3.5×)†
T‡		+ DNP§	Thyroidectomized rats	
			No acceptor	+ DNP§
	100	697	65	454
	88	590	47	485
	73	565	38	433
	79	671	39	530
	68	579	55	458
	100	494	37	511
Average of 6 experiments	85	599 (7×)†	47	478 (10×)†

The reaction vessels contained ATP, phosphate buffer, pH 7.4, $MgSO_4$, cytochrome *c*, and glutamate in the amounts recorded in Table I, and isotonic sucrose to a final volume of 3.0 ml. The phosphate-accepting system contained 50 μmoles of glucose and an excess of yeast hexokinase; temperature, 30°. Q_{O_2} (N) is calculated from the first 20 minutes of respiration following an 8 minute equilibration period. The thyroidectomized rats were maintained for at least 30 days to deplete their body stores of thyroid hormone; during this time they were fed a synthetic ration to avoid dietary sources of thyroxine. After sacrifice, gross examination of the thyroid region was made to ascertain complete removal.

* Mitochondria from 200 mg. of fresh liver in each flask.

† The values in parentheses represent the degree of stimulation by phosphate acceptor or DNP.

‡ Mitochondria from 300 mg. of fresh liver in each flask.

§ At 5×10^{-5} M.

consumed oxygen more than twice as rapidly as did the normal mitochondria. However, they still displayed a strong phosphate acceptor effect.

In a separate series of experiments, a comparison was made of the respiration rate of mitochondria from normal and thyroidectomized rats (Table III, Series T). DNP, at 5×10^{-5} M, was used as the respiration accelerator.

This concentration had previously been found to enhance respiration to the same maximum as did the hexokinase system (12). The finding that mitochondria from thyroidectomized rats had a lower rate of respiration and a greater degree of acceleration by DNP than did the controls lends support to the hypothesis (1) that normally occurring concentrations of thyroid hormone uncouple an oxidative phosphorylation reaction. The higher rate of respiration in the control experiments of Series T as compared with Series H results from the higher mitochondrial concentration. With the small amounts of mitochondria employed in these experiments, respiration is not quite directly proportional to the amount of mitochondria.

TABLE IV

Phosphate Acceptor Effect on Liver Mitochondria from Normal and Thyrotoxic Rats in Presence of Fluoride

Additions	Normal		Thyrotoxic	
	Q_{O_2} (N)*	P:O	Q_{O_2} (N)*	P:O
No acceptor	57		168	
~P "	380 (7×)	3.1	494 (4×)	2.1
No " + 0.0033 M F^-	50		77	
~P " + 0.0033 " "	195 (4×)	3.4	230 (3×)	2.1

Experimental conditions same as for Table III, Series H, except that 10 μmoles of F^- were added where indicated. The results are the average of two experiments. The numbers in parentheses refer to the degree of stimulation by acceptor.

* Calculated on the basis of the first 30 minutes of respiration following an 8 minute equilibration period.

Fluoride, which is known to inhibit a variety of phosphatases, inhibits the respiration of thyrotoxic mitochondria in the absence of phosphate acceptor, but has relatively little effect on normal mitochondria (Table IV). In the presence of hexokinase and glucose, fluoride is equally inhibitory to both types of mitochondria.

Although it had previously been found that KCl-washed particles (2) or KCl-treated mitochondria (10) from thyrotoxic rats exhibit lower P:O ratios than preparations from normal animals, there are complications which might tend to invalidate P:O ratios obtained with these types of preparations (see "Discussion"). It is therefore of importance to emphasize that the decreased P:O ratios reported here were obtained with mitochondria which had not been exposed to extraneous salt during their isolation from other cellular fractions. When it had been demonstrated that thyrotoxic mitochondria had a lowered over-all efficiency of oxidative phosphorylation, an attempt was made to determine the locus of action of the hormone.

The substrate phosphorylation accompanying the oxidation of α-ketoglutarate has been shown by Hunter (14) to be insensitive to 2,4-dinitrophenol in the presence of large concentrations of fluoride. This technique has been used to determine whether the substrate phosphorylation is "uncoupled" in the mitochondrial preparation from thyrotoxic rats (Fig. 1). In both normal and thyrotoxic preparations, increasing levels of DNP lowered the P:O ratio for α-ketoglutarate to a value approximating 1. Although the thyrotoxic rat liver mitochondria appeared to be somewhat

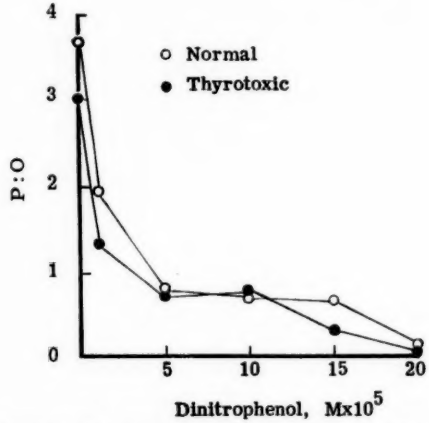


FIG. 1. Comparison of efficiency of substrate phosphorylation accompanying the oxidation of α-ketoglutarate by normal and thyrotoxic rat liver mitochondria. The reaction vessels contained ATP, phosphate buffer, pH 7.4, $MgSO_4$, and cytochrome *c* in the amounts recorded in Table I; 30 μmoles of α-ketoglutarate, 30 μmoles of malonate, 120 μmoles of KF, 50 μmoles of glucose, hexokinase, mitochondria from 0.5 gm. of fresh liver, and isotonic sucrose to a final volume of 3.0 ml. 0.2 ml. of 5 N KOH in the center well; gas phase, air; temperature, 30°.

more sensitive to higher concentrations of DNP than did the normal, it is clear that this substrate phosphorylation is not strikingly uncoupled.

Recently it has been demonstrated that a phosphorylation accompanies the oxidation of cytochrome *c* reduced by ascorbic acid or adrenaline (8, 15). The results of experiments comparing the P:O ratio for the oxidation of reduced cytochrome *c* by liver mitochondria from normal and thyrotoxic rats are shown in Table V. The quantities of mitochondria and substrates used were those previously established (8) as optimal. With adrenaline as the substrate, the mitochondria from thyrotoxic rats fixed phosphate nearly as efficiently as did the control preparations. A greater difference was found when ascorbic acid was employed to reduce cytochrome *c*, but

this may be related to the fact that ascorbic acid itself can cause uncoupling (16). It is probable that the results with adrenaline are more useful in defining primary effects of the thyroid hormone, and that the low values obtained with ascorbic acid result from a combination of "uncoupling effects."

The precise site at which ferricyanide taps into the electron transport system in intact mitochondria is not known. However, it has been established that it is below the level at which antimycin A impinges on the electron transport enzyme sequence (7). The phosphorylation associated with the oxidation of D-β-hydroxybutyrate[2] to acetoacetate in the presence

TABLE V

Phosphorylation Associated with Oxidation of Reduced Cytochrome c in Liver Mitochondria from Normal and Thyrotoxic Rats

Substrate	No. of experiments	Normal		Thyrotoxic	
		O uptake	P:O	O uptake	P:O
		µatoms		*µatoms*	
Adrenaline.....	14	7.9*	0.72 ± 0.06†	9.1*	0.50 ± 0.1
Ascorbic acid.....	10	14.1‡	0.68 ± 0.08	14.8‡	0.27 ± 0.1

The reaction vessels contained 6 µmoles of ATP, 30 µmoles of phosphate buffer, pH 7.4, 30 µmoles of $MgSO_4$, 0.3 µmole of cytochrome *c*, 10 µmoles of ascorbic acid or 30 µmoles of adrenaline, 20 µmoles of KF, 50 µmoles of glucose, excess yeast hexokinase, mitochondria from 0.2 or 0.5 gm. of fresh liver, and isotonic sucrose to a final volume of 3.0 ml. 0.2 ml. of 5 N KOH in center well; gas phase, air; temperature, 30°. All flasks were run in duplicate.

* Per 10 minutes per 2 mg. of N.

† Standard deviation.

‡ Per 20 minutes per mg. of N.

of ferricyanide as electron acceptor was compared in mitochondria from normal, thyrotoxic, and thyroidectomized rats. The results, summarized in Table VI, indicate that the thyrotoxic mitochondria consistently yield a P:O ratio about 50 per cent lower than the normal preparation. In four experiments with thyroidectomized rats, no differences from normal were observed. During many of the experiments it was observed that the rate of reduction of ferricyanide by thyrotoxic rat liver mitochondria was considerably lower than that observed for the normal preparation. This seemed inconsistent with the usual increased oxidation rate of hyperthyroid tissues. Additions of various coenzymes *in vitro* were made in an attempt to restore to normal the reduction of ferricyanide by thyrotoxic

[2] The configuration of levorotatory β-hydroxybutyric acid has been established as D (29). In a previous paper (4) we incorrectly used the designation L.

rat liver mitochondria. The obvious addition of diphosphopyridine nucleotide proved a fortunate one. Additions of 2 to 4 μmoles of DPN increased the reduction of ferricyanide by thyrotoxic rat liver mitochondria 60 to 70 per cent, whereas this increase in the normal animal was only 30

TABLE VI

Phosphorylation Accompanying Oxidation of D-β-Hydroxybutyrate to Acetoacetate with Ferricyanide As Electron Acceptor

Condition of rat	No. of experiments	$Fe(CN)_6^{=}$ reduction	P:2Fe(CN)$_6^{=}$
		μmoles	
Normal	18	15.0	1.09 ± 0.15
Thyrotoxic	18	13.1	0.53 ± 0.24
Thyroidectomized	4	15.2	1.07 ± 0.27

The reaction vessels contained 6 μmoles of ATP, 40 μmoles of phosphate buffer, pH 7.4, 15 μmoles of $MgSO_4$, 50 μmoles of $KHCO_3$, 50 μmoles of ferricyanide, 30 μmoles of D-β-hydroxybutyrate, 50 μmoles of glucose, hexokinase, mitochondria from 0.5 gm. of fresh liver, and isotonic sucrose to a final volume of 3.0 ml. Gas phase, 95 per cent N_2, 5 per cent CO_2; temperature, 30°. Ferricyanide reduction was measured by direct colorimetry with a 420 mμ filter. Inorganic phosphate was determined as described by Copenhaver and Lardy (7). $Fe(CN)_6^{=}$ reduction is expressed in micromoles per 20 minutes per 2 mg. of N.

TABLE VII

Effect of Addition in Vitro of DPN on Oxidation of D-β-Hydroxybutyrate with Ferricyanide As Electron Acceptor

DPN added	Normal		Thyrotoxic	
	Δ $Fe(CN)_6^{=}$	P:2Fe(CN)$_6^{=}$	Δ $Fe(CN)_6^{=}$	P:2Fe(CN)$_6^{=}$
μmoles	*μmoles*		*μmoles*	
None	13.4	1.29	9.8	0.68
2	16.2	1.18	14.4	0.79
4	17.4	1.17	16.0	0.79

The conditions of the experiments were the same as for Table VI. The results reported are the averages of five experiments. $Fe(CN)_6^{=}$ reduction is expressed in micromoles per 20 minutes per 2 mg. of N.

per cent (Table VII). The addition of DPN, however, did not restore the phosphorylating ability of the thyrotoxic tissues.

Since conflicting reports on the content of pyridine nucleotides in hyperthyroid tissues have appeared (17, 18), the total pyridine nucleotide content of mitochondria was determined by the method of Feigelson, Williams, and Elvehjem (19). Mitochondria were isolated as usual from normal and

thyrotoxic rat livers in isotonic sucrose, and the mitochondrial suspensions were then extracted with trichloroacetic acid and hydrogen peroxide. The pyridine nucleotides from the extracts were adsorbed on activated charcoal, eluted with 10 per cent pyridine, and measured spectrophotometrically in the reduced form. The hyperthyroid rat liver mitochondria had about 40 per cent less pyridine nucleotide than did the preparation from normal rats (Table VIII).

The low pyridine nucleotide concentration in the thyrotoxic rat liver mitochondria may result from a variety of circumstances, causing either decreased synthesis or increased destruction. An attempt was made to test at least some of these possibilities. Injecting thyrotoxic rats with

TABLE VIII

Comparison of Total Pyridine Nucleotides Present in Normal and Thyrotoxic Rat Liver Mitochondria

Pyridine nucleotide per mg. of mitochondrial N		
Normal	Thyrotoxic	Thyrotoxic + niacinamide*
γ	γ	γ
15.0	9.5	10.0
12.3	7.7	7.9
13.6	7.1	6.7
Average....13.6	8.1	8.2

The determinations were run in duplicate.

* 2 mg. per day for 5 days.

2 mg. of niacinamide daily for 5 days did not enhance the liver mitochondrial content of pyridine nucleotides (Table VIII), nor did it affect the response of the mitochondria to added DPN in the β-hydroxybutyrate-ferricyanide system. The ability of normal and thyrotoxic nuclei to synthesize DPN from nicotinamide mononucleotide[3] and ATP was also tested. The enzyme was prepared according to Hogeboom and Schneider (20) as the protein soluble in 1 M NaCl and insoluble in 0.17 M NaCl. No significant difference in the rate of DPN synthesis could be detected. In three experiments an average of 12.1 and 10.7 μmoles of DPN was synthesized per mg. of enzyme nitrogen per hour by the normal and thyrotoxic preparation, respectively. Thus at least one of the enzymes required for synthesis of DPN seems to be present in normal amounts in the thyrotoxic liver.

[3] This compound was kindly supplied by Dr. Gerhard Plaut.

DISCUSSION

For studies of oxidative phosphorylation efficiency, with glucose-hexokinase as the phosphate-trapping system, the importance of isolating mitochondria in sucrose cannot be overemphasized. It is known that the submicroscopic particulate fraction (microsomes) of liver cells contains the phosphatase which specifically hydrolyzes glucose-6-phosphate at neutral pH (21). It is furthermore known that salts added to homogenates in sucrose solution cause coagglutination of the submicroscopic particles and the mitochondria (22, 23). The presence of this source of glucose-6-phosphatase in a system in which phosphate is being trapped as glucose-6-phosphate is particularly undesirable when comparisons are being made of phosphorylating efficiency in mitochondria from animals under different treatment. There is an increased concentration of glucose-6-phosphatase in the livers of diabetic rats (24, 25), fasted rats (26), and thyrotoxic rats (unpublished experiments). Contamination of mitochondria by a portion of the microsomal material might therefore cause a greater *apparent* decrease in P:O ratios of thyrotoxic preparations than of the controls.

In experiments to be published in connection with the study of glucose-6-phosphatase in thyrotoxicity, it has been found that the Schneider procedure (5) removes the microsomes from thyrotoxic mitochondria as effectively as it does from the mitochondria of normal animals. Therefore, the effects of excess thyroid hormone in the intact animal on phosphorylation efficiency seem to be direct. This conclusion is supported by the uncoupling effect of thyroxine and triiodothyronine added *in vitro* to mitochondria (2–4, 10). We have previously pointed out (1, 2, 4, 13, 27) that the decreased efficiency of oxidative phosphorylation in thyrotoxic mitochondria and the decreased control of respiration are a sufficient explanation of the decreased efficiency of work performance and the increased basal metabolism of the hyperthyroid patient.

The experiments reported here do not disclose a specific site of uncoupling by an excess of the thyroid hormone. It is clear that thyrotoxicity does not result in significant uncoupling of the substrate phosphorylation associated with ketoglutarate oxidation. It caused only a slight uncoupling of the phosphorylation resulting from adrenaline oxidation linked through added cytochrome *c* (8), and a marked uncoupling of the phosphorylations occurring between DPNH and ferricyanide. Since the latter oxidation occurs with a P:$2Fe(CN)_6^{=}$ slightly greater than 1 (Tables VI and VII), there is a possibility that two phosphorylation sites are involved and that one of these may be relatively more susceptible to thyroid hormone than the other. This possibility is being investigated.

Our original proposal for the mode of action of the thyroid hormone (1, 2) predicted that, at normal concentrations, the hormone exerts its

effect at one specific step in the phosphorylating electron transport sequence. This view has recently been challenged (10) because of the finding that thyroxine may depress P:O ratios to zero. However, it has been emphasized (4, 13) that the specificity would not be expected to be retained at the toxic levels of the hormone. The selectivity of DNP at low concentrations is also abolished by increasing the concentration several fold ((14) and (Fig. 1)). And it may be pertinent to cite the experiments of Kandler (28), who finds low concentrations of the classic uncoupler, DNP, to exert a "beneficial" effect in stimulating growth of maize seedling roots.

SUMMARY

Oxidation and phosphorylation reactions have been studied with mitochondria isolated from livers of normal and thyrotoxic rats and in some cases from thyroidectomized rats.

Thyrotoxicity decreased the over-all efficiency of oxidative phosphorylation. It also decreased the control of respiration exerted by phosphate acceptor concentration. This control mechanism appeared to be more efficient in mitochondria from thyroidectomized rats than from normal rats.

The substrate phosphorylation accompanying the oxidation of α-ketoglutarate was not sensitive to the thyroid hormone.

The phosphorylation accompanying the oxidation of cytochrome *c* reduced by adrenaline is slightly lowered in thyrotoxicity. When ascorbic acid was employed to reduce cytochrome *c*, the thyrotoxic preparation had a much lower P:O ratio.

Thyrotoxicity depressed the phosphorylations accompanying the oxidation of D-β-hydroxybutyrate to acetoacetate with ferricyanide as the electron acceptor. The decreased rate of this oxidation in thyrotoxic mitochondria could be greatly enhanced by added DPN. The pyridine nucleotide content of thyrotoxic mitochondria was lower than normal, but ability of nuclear extracts to synthesize DPN from nicotinamide mononucleotide and ATP was not altered.

BIBLIOGRAPHY

1. Lardy, H. A., in Wolterink, L. F., The biology of phosphorus, Lansing, 131 (1952).
2. Lardy, H. A., and Feldott, G., *Ann. New York Acad. Sc.*, **54,** 636 (1951).
3. Feldott, G., *Federation Proc.*, **12,** 414 (1953).
4. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **204,** 435 (1953).
5. Schneider, W. C., *J. Biol. Chem.*, **176,** 259 (1948).
6. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29,** 379 (1946).
7. Copenhaver, J. H., Jr., and Lardy, H. A., *J. Biol. Chem.*, **195,** 225 (1952).
8. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **210,** 903 (1954).

XUM

9. Aebi, H., and Abelin, I., *Biochem. Z.*, **324**, 364 (1953).
10. Hoch, F. L., and Lipmann, F., *Proc. Nat. Acad. Sc.*, **40**, 909 (1954).
11. Niemeyer, H., Crane, R. K., Kennedy, E. P., and Lipmann, F., *Federation Proc.*, **10**, 229 (1951); *Bol. soc. biol. Santiago Chile*, **10**, 54 (1953).
12. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195**, 215 (1952).
13. Lardy, H. A., and Maley, G. F., *Recent Progress Hormone Res.*, **10**, 129 (1954).
14. Hunter, F. E., Jr., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1**, 297 (1951).
15. Lehninger, A. L., ul Hassan, M., and Sudduth, H. C., *J. Biol. Chem.*, **210**, 911 (1954).
16. Feldott, G., and Lardy, H. A., *Federation Proc.*, **11**, 210 (1952).
17. Katzenelbogen, E., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **141**, 611 (1941).
18. Galeone, A., Leve, E., and Segne, G., *Folia endocrinol.*, **4**, 595 (1951).
19. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **185**, 741 (1950).
20. Hogeboom, G. H., and Schneider, W. C., *J. Biol. Chem.*, **197**, 611 (1952).
21. Hers, H. G., Berthet, J., Berthet, L., and de Duve, C., *Bull. Soc. chim. biol.*, **33**, 21 (1951).
22. Hogeboom, G. H., and Schneider, W. C., *J. Biol. Chem.*, **186**, 417 (1950).
23. Huseby, R. A., and Barnum, C. P., *Arch. Biochem.*, **26**, 187 (1950).
24. Ashmore, J., Hastings, A. B., and Nesbett, F. B., *Proc. Nat. Acad. Sc.*, **40**, 673 (1954).
25. Parks, R. E., Jr., Thesis, University of Wisconsin (1954).
26. Weber, G., and Cantero, A., *Science*, **120**, 851 (1954).
27. Lardy, H. A., Brookhaven symposia in biology, No. 7, 90 (1954).
28. Kandler, O., *Z. Naturforsch.*, **5b**, 338 (1950).
29. Lemieux, R. U., and Giguere, J., *Canad. J. Chem.*, **29**, 678 (1951).

ENZYMATIC SYNTHESIS AND PROPERTIES OF 5-PHOSPHORIBOSYLPYROPHOSPHATE*

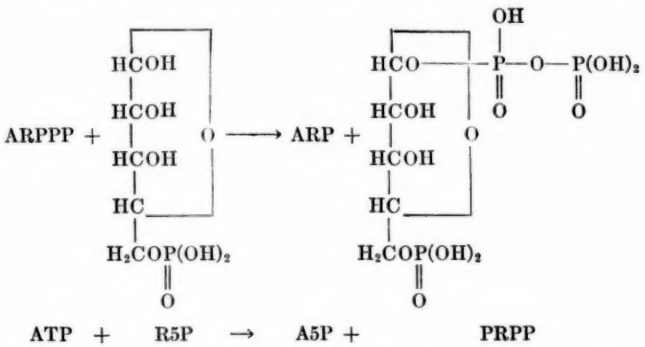
By ARTHUR KORNBERG, IRVING LIEBERMAN, AND ERNEST S. SIMMS

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, November 22, 1954)

Investigations of the mechanism of pyrimidine nucleotide formation from orotic acid led to the isolation and characterization of an activated ribose phosphate derivative which serves as an intermediate in the overall process. In earlier communications (1) we have described briefly the enzymatic synthesis of this new intermediate, identified as PRPP[1] (equation (1)), and the occurrence of enzymes which condense pyrimidines and purines with PRPP to form the respective nucleotides (equation (2)).

(1)



(2) Pyrimidine (or purine) + PRPP $\rightleftharpoons$ pyrimidine (or purine) nucleotide + PP

This report deals with the purification and properties of the enzyme which catalyzes the formation of PRPP and some of the properties of PRPP. Succeeding reports will consider the enzymatic synthesis of pyrimidine nucleotides (2), purine nucleotides (3), and nucleoside di- and triphosphates (4).

* This investigation was supported by grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

[1] The abbreviations used are 5-phosphoribosylpyrophosphate, PRPP; adenosine triphosphate, ATP or ARPPP; ribose-5-phosphate, R5P; adenosine-5′-phosphate, A5P or ARP; inorganic pyrophosphate, PP; adenosine diphosphate, ADP; orotidine-5′-phosphate, O5P; uridine-5′-phosphate, U5P; tris(hydroxymethyl)aminomethane, Tris.

Methods

Materials—The sodium salt of R5P was prepared from the barium salt (product of the Schwarz Laboratories, Inc.) by decomposition with sodium sulfate. A5P and ADP were products of the Sigma Chemical Company and ATP of the Pabst Brewing Company. Adenine and orotic acid were obtained from Dougherty Chemicals. Ribose-1-phosphate was prepared by phosphorolysis (5) of inosine (Nutritional Biochemicals Corporation) with liver nucleoside phosphorylase[2] coupled to xanthine oxidase;[2] it was purified by anion exchange chromatography (6). ATP^{32} (ATP labeled in the two terminal phosphate groups) was prepared by aerobic phosphorylation of A5P with rat liver mitochondria[3] (7) and purified by the ion exchange chromatographic procedure of Cohn and Carter (8) and Kornberg and Pricer (9).

2-Deoxyribose-5-phosphate was prepared from 2′-deoxyadenosine-5′-phosphate (California Foundation for Biochemical Research) as follows: 1.6 ml. of a 0.04 M solution of the nucleotide was acidified to pH 2, heated at 100° for 5 minutes, neutralized, adsorbed on a Dowex 1 column (2 per cent cross-linked, chloride form, 3 cm. × 1 sq. cm.), and eluted with 0.005 N HCl-0.01 M KCl. The fractions between 15 and 23 resin bed volumes contained 97 per cent of the adsorbed deoxypentose and an equivalent amount of organic phosphate. The acid lability of the compound (94 to 98 per cent hydrolysis after 30 minutes at 100° in 1 N HCl) agrees with published observations (10).

Orotic acid (4,7-C^{14})[4] was prepared from 1,4-C^{14}-aspartic acid by a modification of the method of Nyc and Mitchell (11). Labeled aspartic acid was prepared as follows: Ethylene dibromide (1.7 gm.) was allowed to react with C^{14}-NaCN (10 mmoles) by a microadaptation[5] of a procedure of Vanino (12), producing 1,4-C^{14}-succinic acid, which was then partially purified by treatment with a cation exchange resin (Dowex 50 (H^+)). The percolate was evaporated to dryness and the residue was dissolved in water and neutralized. The succinate was quantitatively oxidized by succinoxidase (13) to an equilibrium mixture of fumarate and malate. The latter mixture was then converted to L-aspartate in the presence of excess ammonia by an aspartase-fumarase preparation from *Escherichia coli* (14). L-Aspartic acid was purified by chromatography on Dowex 50 (H^+), concentrated *in vacuo*, and crystallized at pH 3.0 by the addition of alcohol.

Acetone powders of liver were prepared as follows: The fresh tissue was

[2] Kindly furnished by Dr. M. Friedkin.

[3] Kindly furnished by Dr. F. E. Hunter.

[4] The numbering of the pyrimidine ring follows recent usage by "Chemical abstracts;" orotic acid is 6-carboxyuracil.

[5] We are indebted to Dr. D. Shemin for these details.

homogenized in 5 to 10 volumes of acetone (chilled to −10°). The residue, collected on a Büchner funnel, was homogenized again in cold acetone, collected, and dried in air. The powders were stored at −10°.

Determinations—ADP and ATP were determined spectrophotometrically (15, 16). A5P was determined with Schmidt's deaminase by Kalckar's method (17). PRPP was determined as described below (Assay B, Stage II). Orthophosphate was determined by the method of Fiske and Subbarow (18), acid-labile phosphate was the orthophosphate liberated after hydrolysis for 10 minutes in 1 N HCl at 100°, and total phosphate was obtained as orthophosphate after ashing with a sulfuric acid-nitric acid mixture. Reducing sugar was determined colorimetrically (19) with D-ribose as a standard. Pentose was determined by the Mejbaum procedure (20) with a 40 minute heating period and A5P as a standard. Protein was determined by the phenol method of Lowry *et al.* (21). Ion exchange chromatography was carried out at 2° with an automatic fraction collector on Dowex 1 (2 per cent cross-linked, 200 to 400 mesh, chloride form) columns according to Cohn (22). P^{32} was measured in solution in a dish under a Geiger-Müller tube. C^{14}-containing samples were plated as thin layers on metal disks and measured in a gas flow counter. Self-absorption corrections were applied where indicated.

Enzyme Assays—The activity responsible for equation (1) can be determined in a variety of ways. Three methods which have provided sensitive and reliable assays for the enzyme will be described here.

Assay A. $C^{14}O_2$ Evolution from Carboxyl-Labeled Orotate—This assay requires the addition, in excess, of O5P pyrophosphorylase and decarboxylase which carry out reactions (3) and (4), respectively. Under these

$$\text{Orotate} + \text{PRPP} \rightleftarrows \text{O5P} + \text{PP} \tag{3}$$

$$\text{O5P} \rightarrow \text{U5P} + CO_2 \tag{4}$$

conditions the amount of $C^{14}O_2$ liberated is an accurate measure of the amount of PRPP produced in the system. The incubation mixture (1.0 ml.) contained 0.02 ml. of ATP (0.04 M), 0.02 ml. of phosphopyruvate (0.1 M), 0.1 ml. of R5P (0.025 M), 0.05 ml. of 4,7-C^{14}-orotate (0.01 M, 1.4×10^5 c.p.m. per μmole), 0.05 ml. of potassium phosphate buffer (1 M, pH 7.0), 0.03 ml. of $MgCl_2$ (0.1 M), 0.01 ml. of pyruvate phosphokinase (16), 0.1 ml. of yeast autolysate (2), and less than 0.2 unit of enzyme. The mixture was incubated at 30° for 1 hour in an evacuated Thunberg tube containing 0.5 ml. of 0.2 N KOH (with a drop of 0.04 per cent thymol blue) in the bulb. To stop the reaction and acidify the reaction mixture, about 4 drops of an H_2SO_4-thymol blue solution (2 parts of 50 per cent H_2SO_4, 1 part of 0.04 per cent thymol blue) were cautiously admitted into the main space of the Thunberg tube through the side arm. The tube,

still under vacuum, was incubated in a 65° oven for 30 minutes and then cooled. 0.1 ml. of the KOH solution in the bulb was removed to a planchet and dried and the radioactivity was measured. Under these conditions (with a self-absorption of about 50 per cent), 3500 c.p.m. represented total decarboxylation of the 0.5 μmole of orotate in the reaction mixture. Proportionality of enzyme addition to $C^{14}O_2$ release was obtained when 30 per cent or less of the orotate was decarboxylated. 1 unit of enzyme is defined as the amount causing the formation of 1 μmole of PRPP (as indicated by the release of 1 μmole of $C^{14}O_2$), or the removal of 1 μmole of orotate (Assay B, below), or the removal of 1 μmole of ATP^{32} (Assay C, below), per hour, and specific activity is expressed as units per mg. of protein.

Assay B. Orotate Removal Measured Spectrophotometrically—This assay is best carried out in two stages; the first involving the formation of PRPP and the second its quantitative utilization in the removal of orotic acid as in equation (3).

Stage I—The incubation mixture (1.0 ml.) contained 0.03 ml. of ATP (0.04 M), 0.1 ml. of R5P (0.025 M), 0.02 ml. of potassium phosphate buffer (1 M, pH 7.4), 0.02 ml. of $MgCl_2$ (0.1 M), 0.02 ml. of glutathione (0.5 M), 0.05 ml. of KF (1 M), and about 1 unit of enzyme. After incubation for 20 minutes at 36°, the mixture was heated for 1 minute in a boiling water bath to inactivate the enzyme, immediately cooled, and centrifuged if necessary.

Stage II—The incubation mixture (1.0 ml.) in a quartz cuvette contained 0.02 ml. of orotate (0.01 M), 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), 0.2 ml. of O5P pyrophosphorylase (ethanol fraction) (2), and an aliquot from Stage I containing about 0.03 μmole of PRPP. The conversion of orotic acid to O5P or U5P results in a decrease in optical density at 295 mμ; the molar absorption coefficient for the density decrease of the latter conversion is 3950. With the indicated amount of O5P pyrophosphorylase fraction, the utilization of 0.03 μmole of PRPP is usually complete in 10 minutes when measured in the Beckman DU spectrophotometer at room temperature.

Assay C. ATP^{32} Removal—This assay depends on transfer of the P^{32}-labeled terminal phosphate groups of ATP to a compound that is not adsorbable by Norit (23). The incubation mixture (0.4 ml.) contained 0.02 ml. of ATP^{32} (0.02 M, 3×10^4 c.p.m. per μmole), 0.04 ml. of R5P (0.025 M), 0.02 ml. of potassium phosphate buffer (1 M, pH 7.4), 0.01 ml. of $MgCl_2$ (0.1 M), 0.02 ml. of glutathione (0.5 M), 0.04 ml. of KF (1 M), and about 0.2 unit of enzyme. After incubation for 30 minutes at 36°, 0.8 ml. of 3.5 per cent perchloric acid and then 0.2 ml. of a 10 per cent suspension of Norit A were added to the reaction mixture. After 2 to 5 minutes the

mixture was filtered and 0.5 ml. of the filtrate was pipetted into a dish for radioactivity measurement. With crude enzyme fractions, an incubation mixture lacking R5P served as a control for interfering activities such as that of ATPase. The use of fluoride provides a means of reducing ATPase activity.

Results

Purification of Enzyme—All operations were carried out at 0–3°. 4 gm. of pigeon liver acetone powder were suspended in 40 ml. of Tris buffer (0.02 M, pH 8.0) and stirred occasionally during a 10 minute interval. The residue was removed by centrifugation in a Servall (SS-1) centrifuge at 8000 × *g* for 5 minutes. The supernatant fluid may be either clear or opalescent (acetone powder extract, Table I).

TABLE I

Purification of Enzyme

Step	Units per ml.*	Total units*	Protein	Specific activity
			mg. per ml.	*units per mg. protein*
1. Acetone powder extract	18.0	612	19.6	0.9
2. Low pH precipitation	16.4	558	3.00	5.5
3. Aluminum hydroxide gel eluate	8.1	275	0.33	24.5

* Assay B was used in this run. Essentially similar results were obtained with the other assays.

To 34 ml. of extract were added 51 ml. of water and then, slowly, 17 ml. of potassium acetate buffer (1 M, pH 5.4). After 5 minutes, the precipitate was collected by centrifugation (as above). It was dissolved in 7.0 ml. of Tris buffer (0.1 M, pH 8.0) and diluted to 30 ml. with water. The pH was adjusted to 6.8 with 0.1 N HCl (about 1.3 ml. required). The volume was made up to 34 ml. (low pH fraction).

For the last step enough aluminum hydroxide gel Cγ was used to adsorb 85 to 90 per cent of the enzyme. 3.75 ml. of aged gel (24) (15 mg. of dry weight per ml.) were centrifuged and the supernatant fluid was discarded. 30 ml. of isoelectric fraction were thoroughly mixed with the gel, left in ice for 5 minutes, and then centrifuged. The supernatant fluid was discarded and the gel precipitate was washed with 30 ml. of potassium phosphate buffer (0.05 M, pH 6.85). The enzyme was then eluted from the gel with 30 ml. of potassium phosphate buffer (0.10 M, pH 6.85) (aluminum hydroxide gel eluate).

The acetone powders were stored at −10° and showed no significant

loss in activity over a 2 month period. The Cγ gel eluate retained 65 to 70 per cent of its activity after storage for 2 months at −10° provided glutathione (0.05 M) was present; in the absence of glutathione less than 5 per cent of the activity remained.

Distribution of Enzyme—The enzyme appears to be present also in chicken and mammalian livers and in bacteria. Extracts of acetone powders of chicken, mouse, and rat livers, and of an 18 hour culture of *E. coli* B cells (ground with alumina (25)) had the following specific activities (Assay B): chicken, 0.29; mouse, 0.04; rat, 0.01; and *E. coli*, 0.34. An

TABLE II

Balance Study of PRPP Synthesis

The experimental incubation mixture (10.0 ml.) was the same as that described for Assay B, Stage I (see the text), except that ATP^{32} (2.2×10^4 c.p.m. per μmole) was used. The control mixture lacked R5P. 2.0 ml. of the aluminum hydroxide gel eluate fraction containing 0.72 mg. of protein were used. Incubation was at 35° for 60 minutes. The figures in bold-faced type were obtained by chromatography of 8.0 ml. of the reaction mixtures on Dowex 1 columns (6.5 cm. × 1.0 sq. cm.); the eluent was 0.0025 N HCl-0.1 M KCl. The other values were obtained by direct enzymatic assay of aliquots of the incubation mixtures. The results are expressed in micromoles.

	0 min.		60 min.		Δ	
	Control	Experimental	Control	Experimental	Control	Experimental
ATP	13.1	12.5	12.5	1.6	−0.6	−10.9
"			**11.1**	**1.8**		
PRPP	0.0	0.0	0.0	10.9	0.0	+10.9
"				**10.0**		
A5P	0.0	0.0	0.0	9.8	0.0	+9.8
"			**0.0**	**10.2**		

autolysate of dried brewers' yeast and a sonic extract of bakers' yeast were inactive.

Balance Study of Reaction—The stoichiometry of the reaction of ATP with R5P to yield A5P and PRPP (equation (1)) is shown in Table II. In the control sample, lacking R5P, ATP was not removed to any significant extent and the production of A5P and PRPP was not detectable. In the presence of R5P, almost all the added ATP disappeared and was matched by the accumulation of equivalent molar amounts of A5P and PRPP. Values obtained both by enzymatic and ion exchange chromatographic analyses were in good agreement. No ADP was detectable after either 30 or 60 minutes incubation when 65 and 87 per cent, respectively, of the ATP had been consumed. That orthophosphate was not released (from

the ATP^{32} in this reaction) was evident from the absence of any radioactivity in that fraction of the ion exchange chromatogram.

Isolation of PRPP—PRPP was isolated by ion exchange chromatography as a symmetrical zone, appearing after 25 to 30 resin bed volumes of effluent, and was contained within the next 10 to 15 volumes. The early fractions were contaminated by ATP. Analyses of eight fractions selected

TABLE III

Chromatography of PRPP

8.5 ml. of experimental and control incubation mixtures were chromatographed as described (see Table II).

Fraction No.*	Pentose†	Molar ratios (referred to pentose)			Specific activity§
		PRPP	Acid-labile P‡	Total P	
	μmole per ml.				*c.p.m. per μmole*
30	0.051	1.00			20,300
32	0.100	0.84	2.00	3.05	22,500
34	0.107	1.03	1.98	2.96	21,300
36	0.109	1.00	2.01	3.00	22,900
38	0.107	0.95	2.01	3.15	23,200
40	0.101	0.94	2.03	2.95	23,000
42	0.081	0.84	2.10	3.15	23,200
44	0.060		2.15	3.05	22,600
Average molar ratio	1.00	0.94	2.04	3.04	

* Approximately 5.0 ml. per fraction, corresponding to about 1 resin bed volume.

† These values are corrected for any ATP present as determined by the optical density at 260 mμ.

‡ Estimations of orthophosphate by the method of Lowry and Lopez (26) indicated 0.16 μmole or less per μmole of pentose in each fraction. With the Fiske and Subbarow procedure (18), 25 and 40 per cent of the acid-labile phosphate appeared as orthophosphate after color development (at 30°) for 10 and 30 minutes, respectively.

§ The specific activities for the ATP^{32} at zero time and that isolated from the control sample were 22,700 and 21,400, respectively.

from the middle of the PRPP zone and containing approximately 80 per cent of the PRPP contained pentose, enzymatically active PRPP (see Assay B, Stage II, equation (3)), acid-labile P, and total P in molar ratios of 1.00:0.94:2.04:3.04 (Table III). The average of the specific radioactivities of these fractions was 22.4×10^3 as compared with values of 22.7×10^3 and 21.4×10^3, respectively, for the ATP^{32} at zero time and that isolated from the control sample.

A barium salt of PRPP was prepared as follows: ATP^{32} and the components described for Assay B, Stage I on a 100 ml. scale, were incubated

for 75 minutes. After 45, 60, and 75 minutes, PRPP synthesis was estimated to be 103, 92, and 100 μmoles, respectively. The mixture was chromatographed on a 6 cm. × 3.2 sq. cm. column.[6] The fractions between 29 and 46 resin bed volumes were neutralized immediately upon their collection and pooled (366 ml., including washings). 7.5 ml. of glycine buffer (1 M, pH 8.5) and 3 ml. of saturated barium acetate were added and the pH was adjusted to 8.5. Cold ethanol (2 volumes) was added and after 30 minutes at 0° the precipitate was collected by centrifugation, washed with ethanol, and dried in a vacuum desiccator. The salt (118 mg.) was suspended in 4 ml. of water, dissolved by the addition of acid (about 0.3 ml. of 0.5 N HCl lowered the pH to about 2),[7] and decomposed with sulfate (0.3 ml. of 1 M Na_2SO_4) and the supernatant solution was neutralized. The reagents were cold and the operations were rapid. 45 μmoles of PRPP were recovered. Extraction of the precipitate with dilute acid yielded 2.8 μmoles more. The recovery of PRPP from the incubation mixture was thus approximately 50 per cent. From 20 to 30 per cent of the radioactivity in the barium salt did not appear in solution. Based on a molecular weight for the barium salt of 729 gm. and without any corrections, the purity of the salt was only 30 per cent.

Reprecipitation of the barium salt from CO_2-free solutions resulted in considerable purification. A crude barium salt (65 mg.) was dissolved in dilute acid and immediately neutralized. The precipitate which formed was washed with 50 per cent ethanol and dried. The salt (24 mg.), on decomposition with sulfate, yielded 18.3 μmoles of PRPP. Assuming that 25 per cent of the PRPP in the barium salt fails to dissolve, the purity of this salt was 75 per cent. In some purified barium salts, ATP (or an ATP-like substance) represented 10 per cent or more of the impurity. Removal of the adenine nucleotide with Norit either was incomplete or led to large losses of PRPP. The major impurities in most cases appeared to be PP and R5P, the decomposition products of PRPP. A preparation free of ultraviolet-absorbing components (<1.0 density unit at 260 mμ per 10 μmoles of PRPP) was obtained by excluding the early fractions of the chromatogram. The molar ratios of inorganic P, acid-labile P, total P, and PRPP to pentose were 0.09, 2.04, 3.02, and 0.70, respectively. Assay with inorganic pyrophosphatase indicated the presence of 5.6 μmoles of PP per 10 μmoles of PRPP. Assuming the presence of an amount of R5P equivalent to that of PP, these two impurities would adequately account for the analytic data.

[6] As compared with the smaller scale run (Table II), 10 times as much reaction mixture was chromatographed on a column only 3 times as large; the separation of PRPP and adenine nucleotides appeared to be less effective.

[7] A large evolution of gas indicated the presence of $BaCO_3$ as an impurity.

Neutral solutions of PRPP have been stored at −15° for 3 months without detectable destruction. At low pH or at elevated temperatures (see below), PRPP decomposition is rapid.

TABLE IV

Decomposition of PRPP

A solution containing 1.45 μmoles of PRPP (P^{32}-labeled, see Table II) in 0.1 M acetate buffer, pH 4.0, was heated at 65°. A solution immediately neutralized without heating served as a control.

Experiment No.	Time of heating	−Δ PRPP	+Δ reducing sugar	+Δ PP
	min.	*μmoles*	*μmoles*	*μmole*
1	10	0.94	0.96	
	40	1.45	1.73	
2	30	1.13	1.07	0.99*

* Determined by ion exchange chromatographic analysis; 0.30 μmole of orthophosphate was also produced.

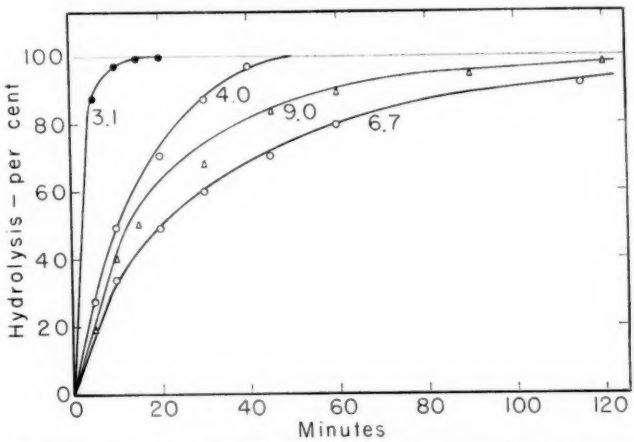


FIG. 1. Decomposition of PRPP at several pH levels. PRPP at a concentration of 0.4 μmole per ml. was incubated in 0.02 M buffer at 65°. The buffers were as follows: formate, pH 3.1, acetate, pH 4.0, glycylglycine, pH 6.7, glycine, pH 9.0.

Structure and Decomposition of PRPP—Heating of PRPP at 65° at pH 4.0 results in a loss of enzymatically active compound (see Assay B, Stage II, equation (3)) and the appearance in equivalent amounts of reducing sugar and inorganic pyrophosphate (Table IV).

The hydrolysis of PRPP at 65° at several pH levels is shown in Fig. 1. At pH 3.1, hydrolysis was virtually complete in 10 minutes, while at neu-

tral pH only 50 per cent was destroyed after 20 minutes. The rate of PRPP decomposition was catalyzed by Mg^{++} and this effect was overcome by agents which bind Mg^{++} (Table V).

The enzymatic decomposition of PRPP has been demonstrated in the reversible pyrophosphorolysis of pyrimidine and purine nucleotides (equation (2)) (2, 3). Nucleotide pyrophosphatase (27) and inorganic pyrophosphatase (28) failed to split (less than 0.005 μmole) PRPP; under comparable conditions, over 25 and 100 μmoles of diphosphopyridine

TABLE V

Mg^{++} Activation of PRPP Decomposition

1 ml. of a 1×10^{-4} M neutral solution of PRPP (in a 12 × 100 mm. glass tube) was heated for 1 minute in a briskly boiling water bath and then immediately placed in ice water. PRPP was assayed as described under "Methods." The concentrations of the indicated additions were $MgCl_2$ 0.002 M, potassium phosphate buffer, pH 7.4, 0.02 M, sodium pyrophosphate, pH 8.0, 0.02 M, Versenate, pH 7.0, 0.005 M. Experiment II differed from Experiment I only in that Tris-maleate buffer, pH 7.4, 0.02 M, was included.

Addition	1 min. hydrolysis (100°)	
	Experiment I	Experiment II
	per cent	*per cent*
None	55	50
Mg^{++}*	78	80
Phosphate	29	
Mg^{++} + phosphate†	18	22
" + pyrophosphate		10
" + Versenate		17

* Heating for 60 minutes at 36° results in a 51 per cent loss of PRPP.

† Heating for 2 minutes at 100° results in a 46 per cent loss of PRPP.

nucleotide and PP, respectively, were hydrolyzed by these enzyme preparations.

Influence of ATP and R5P Concentration on Reaction Rate and Specificity of ATP and R5P for Enzyme—The influence of the concentration of ATP and R5P on the rate of reaction is shown in Fig. 2. No reaction was detectable in the absence of ATP and R5P; the levels at which the rates were half maximal were 4×10^{-4} M and 6×10^{-4} M for ATP and R5P, respectively. ATP at higher concentrations (2×10^{-3} M) appeared to be inhibitory.

ADP (1×10^{-3} M), inosine triphosphate (0.8×10^{-3} M), and uridine triphosphate (0.4×10^{-3} M) either failed to replace ATP or showed less than 2 per cent of the activity of ATP. Ribose-1-phosphate (0.6×10^{-3} M),

2-deoxyribose-5-phosphate (2.5×10^{-3} M), and glucose-6-phosphate (2.5×10^{-3} M) appeared totally inactive in replacing R5P as determined by Assays

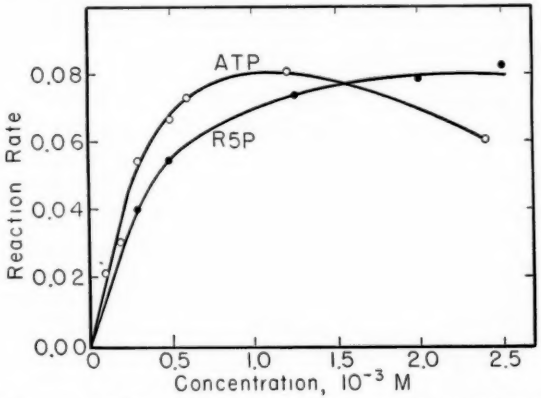


FIG. 2. Influence of ATP and R5P concentration on the rate of PRPP synthesis. The incubation conditions were as described in Assay B, Stage I, except for the varying concentrations of ATP and R5P indicated in the graph. 0.04 ml. of the alumina gel eluate fraction containing about 0.3 unit of enzyme was used. The reaction rate is expressed as units of enzyme activity.

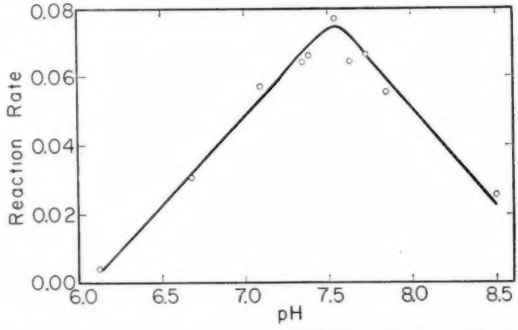


FIG. 3. Influence of the pH on the rate of PRPP synthesis. The incubation conditions were as described in Assay B, Stage I, except for adjustment of the phosphate buffer to the values indicated in the graph. 0.05 ml. of the alumina gel eluate fraction containing about 0.4 unit of enzyme was used. The reaction rate is expressed as units of enzyme activity.

B and C; the action of R5P (2.5×10^{-3} M) was not inhibited by the presence of an equimolar concentration of 2-deoxyribose-5-phosphate.

Effects of Mg^{++} and pH on Reaction—Mg^{++} is essential for the enzyme

activity. In its absence no reaction was observed. The rate was half maximal at a concentration of 0.8×10^{-3} M and maximal at 3×10^{-3} M. The optimal pH for the reaction under standard assay conditions was found to be near 7.5 (Fig. 3). Fluoride did not inhibit the enzyme even at a concentration of 0.1 M.

DISCUSSION

The proposed structure of PRPP is derived from several lines of evidence: (1) utilization of 1 mole of ATP in its synthesis, with the appearance of 1 mole of A5P, but without liberation of ortho P, (2) specificity of R5P and ATP in its synthesis, (3) isolation of a compound, eluted as a symmetrical peak in an ion exchange chromatogram, containing 1 mole of pentose and 3 moles of P of which 2 are acid-labile, (4) decomposition of the compound by very weak acid hydrolysis to liberate one reducing group and 1 mole of inorganic pyrophosphate, and (5) the participation of this compound in the reversible pyrophosphorolysis of pyrimidine and purine nucleoside-5′-phosphates.

In studies on the mechanism of incorporation of adenine into A5P, a 5-phosphoribokinase (29) was described in pigeon liver extracts which catalyzes the reaction

$$\text{ATP} + 2\text{R5P} \rightarrow \text{A5P} + 2 \text{ ribose-1,5-diphosphate}$$

The report contained no evidence for the stoichiometry of the reaction or for the chemical structure of the ribose diphosphate. The similarity of the substrate, product, and enzyme source to those used in our studies suggests that a more thorough inquiry into the ribose diphosphate-forming system is indicated.

The enzymatic synthesis of PRPP may represent a two-stage transfer of phosphate from ATP to R5P or, what appears more likely, a direct pyrophosphorylation of the substrate. The latter mechanism, if correct, would be unique, since previously described reactions such as the pyrophosphorolysis of coenzymes (30) are reversible systems in which the adenyl rather than the phosphate group is transferred. Maas and Lipmann (31) recently proposed, on the basis of isotopic evidence, that a direct pyrophosphorylation of the enzyme involved in pantothenate formation is a step in the over-all scheme. A more direct demonstration of this mechanism is needed.

The metabolic functions of PRPP form the basis for the succeeding reports on a pathway of synthesis of pyrimidine and purine ribonucleotides. It is possible that reaction schemes leading to the formation of other *N*-ribosyl compounds, such as the ribotide of acyclic purine precursors, and

of nicotinamide may also utilize PRPP as an intermediate.[8] The question as to whether a 2-deoxy analogue of PRPP may provide a means for condensing with pyrimidine and purine bases to form deoxyribonucleotides is also worthy of investigation.

SUMMARY

1. Enzymatic studies of pyrimidine and purine nucleotide synthesis have led to the discovery of a new intermediate, 5-phosphoribosylpyrophosphate (PRPP).

2. An enzyme has been partially purified from pigeon liver which catalyzes the formation of PRPP from ribose-5-phosphate (R5P) and ATP.

$$\text{ATP} + \text{R5P} \rightarrow \text{adenosine-5'-phosphate} + \text{PRPP}$$

This enzyme appears to be present also in mammalian liver and in bacteria.

3. ATP and R5P are specific in this reaction; Mg^{++} is also required.

4. PRPP has been isolated and characterized, and some of its properties have been described.

BIBLIOGRAPHY

1. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2027 (1954). Lieberman, I., Kornberg, A., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2844 (1954).
2. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 403 (1955).
3. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 417 (1955).
4. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 429 (1955).
5. Kalckar, H. M., *J. Biol. Chem.*, **167,** 477 (1947).
6. Rowen, J. W., and Kornberg, A., *J. Biol. Chem.*, **193,** 497 (1951).
7. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191,** 485 (1951).
8. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72,** 4273 (1950).
9. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **191,** 535 (1951).
10. Racker, E., *J. Biol. Chem.*, **196,** 347 (1952).
11. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69,** 1382 (1947).
12. Vanino, L., Handbuch der präparativen Chemie, Stuttgart, **2,** 263 (1937).
13. Stotz, E., and Hastings, A. B., *J. Biol. Chem.*, **118,** 479 (1937).
14. Racker, E., *Biochim. et biophys. acta*, **4,** 211 (1950).
15. Kornberg, A., *J. Biol. Chem.*, **182,** 779 (1950).
16. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193,** 481 (1951).
17. Kalckar, H. M., *J. Biol. Chem.*, **167,** 445 (1947).
18. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
19. Somogyi, M., *J. Biol. Chem.*, **195,** 19 (1952).

[8] A sample of PRPP has been tested by Buchanan and coworkers and found to be active in the formation of ribotides of acyclic purine precursors. These workers have also isolated a pentose phosphate ester which is involved in purine nucleotide synthesis and has been identified as PRPP (personal communication from Dr. J. M. Buchanan).

20. Mejbaum, W., *Z. physiol. Chem.*, **258,** 117 (1939).
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
22. Cohn, W. E., *J. Am. Chem. Soc.*, **72,** 1471 (1950).
23. Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201,** 235 (1953).
24. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56,** 1117 (1923).
25. McIlwain, H., *J. Gen. Microbiol.*, **2,** 288 (1948).
26. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162,** 421 (1946).
27. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182,** 763 (1950).
28. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **192,** 87 (1951).
29. Saffran, M., and Scarano, E., *Nature*, **172,** 949 (1953). Scarano, E., *Nature*, **172,** 951 (1953).
30. Kornberg, A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 392 (1951).
31. Maas, W. K., cited by Lipmann, F., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 599 (1954).

ENZYMATIC SYNTHESIS OF PYRIMIDINE NUCLEOTIDES. OROTIDINE-5′-PHOSPHATE AND URIDINE-5′-PHOSPHATE*

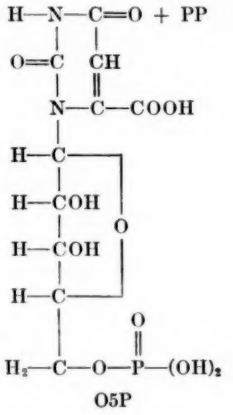
By IRVING LIEBERMAN, ARTHUR KORNBERG, AND ERNEST S. SIMMS

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, November 22, 1954)

Several investigations have established that orotic acid can be a precursor of nucleic acid pyrimidines in bacteria and in animal tissues (1–3). However, the nature of the pathway by which this conversion occurs and the possibility that a non-pyrimidine orotic acid precursor is more directly involved in nucleotide formation required clarification. In preliminary reports (4, 5), we have shown that certain enzymes in yeast convert orotate to U5P[1] in a two-step reaction involving first a condensation with PRPP (6) to form O5P, followed by a decarboxylation. L-Ureidosuccinate and L-dihydroorotate, precursors of orotate (7), proved to be inert in this system.

The purpose of this report is to present the detailed evidence for the

(1) Orotate + PRPP ⇄  + PP

O5P

(2) $$O5P \rightarrow U5P + CO_2$$

* This investigation was supported by grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

[1] The abbreviations used are uridine-5′-phosphate, U5P; 5-phosphoribosylpyrophosphate, PRPP; orotidine-5′-phosphate, O5P; inorganic pyrophosphate, PP; tris-(hydroxymethyl)aminomethane, Tris.

reversible pyrophosphorolysis of O5P (equation (1), O5P pyrophosphorylase) and the decarboxylation of O5P to U5P (equation (2), O5P decarboxylase). Succeeding reports will describe an analogous pyrophosphorylase reaction for the purine bases (8) and the conversion of U5P to uridine di- and triphosphate (9).

Materials

Orotic acid (4,7-C^{14}), PRPP, and P^{32}-labeled PRPP were prepared as previously described (6). Unlabeled orotic acid was obtained from Dougherty Chemicals. Ribose-1,5-diphosphate was prepared according to Klenow (10) from ribose-1-phosphate and glucose-1,6-diphosphate[2] with crystalline phosphoglucomutase.[3] The extent of ribose diphosphate production was calculated from the appearance of glucose-6-phosphate (determined spectrophotometrically (11)). The ribose diphosphate was used as such without further purification.

Orotidine was obtained by growing a mutant strain of *Neurospora crassa* (36601)[4] on a minimal medium supplemented with cytidine sulfate, as described by Michelson *et al.* (12). The mycelial mat was extracted with hot ethanol and treated with barium acetate. The soluble barium salt fraction (23,400 optical density units at 260 mμ) was purified by chromatography on a column of Dowex 1, formate form (200 to 400 mesh, 10 per cent cross-linked, height 7.4 cm., diameter 6.3 cm.). The eluent was formate buffer (0.055 M sodium formate adjusted to pH 3.2 with formic acid). Orotidine, recognized by its absorption spectrum (12), appeared between 23.1 and 30.4 resin bed volumes of eluent and represented 25.4 per cent of the density units applied to the column. Orotidine-containing fractions (1800 ml.) were passed through Dowex 50, hydrogen ion form (resin bed volume of 160 ml.), to remove the sodium ions. Water and formic acid were removed by lyophilization. The residue (96.3 mg.) gave the correct absorption spectrum for orotidine and upon hydrolysis (0.5 N H_2SO_4, 100°) yielded a product with the absorption spectrum of orotic acid.

Malt phosphatase (13) was partially purified as follows: A solution of 4 gm. of malt diastase (Merck) in 120 ml. of water was treated with 29.2 gm. of ammonium sulfate and the precipitate which formed was removed by centrifugation. 16.8 gm. of ammonium sulfate were added to the supernatant fluid. The precipitate was collected by centrifugation and dissolved in 20 ml. of water.

Inorganic pyrophosphatase was partially purified from an extract of dried bakers' yeast with ammonium sulfate and ethanol, as described by Heppel and Hilmoe (14). The ethanol fraction, while essentially free of O5P

[2] Kindly furnished by Dr. L. F. Leloir.
[3] Kindly furnished by Dr. D. H. Brown.
[4] Kindly furnished by Dr. H. K. Mitchell.

pyrophosphorylase, contained *O5P decarboxylase* and could serve as a source of the latter enzyme. Crystalline inorganic pyrophosphatase (15), obtained through the kindness of Dr. G. Perlmann and Dr. M. Kunitz, was free of O5P decarboxylase and pyrophosphorylase.

5'-Nucleotidase, purified from bull semen by the procedure of Heppel and Hilmoe (16), was a gift from Dr. L. A. Heppel.

Determinations

PRPP was estimated spectrophotometrically with orotate and O5P pyrophosphorylase (6). Ion exchange chromatography of PRPP was carried out as previously described (6) on columns of Dowex 1, chloride form (2 per cent cross-linked), at 3°, with a solution of 0.0025 N HCl-0.1 M KCl as eluent. Under these conditions orotic acid and O5P were readily separated, appearing between 5 to 8 and 13 to 21 resin bed volumes of eluent, respectively.

Pentose, phosphate, and protein were estimated as previously described (6).

Enzyme Assays

Assay of O5P Pyrophosphorylase—The assay is based on the decrease in optical density at 295 mμ upon the conversion of orotate to U5P (equations (1) and (2)). The test system contained 0.02 ml. of $MgCl_2$ (0.1 M), 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.03 ml. of sodium orotate (0.01 M), 0.1 ml. of PRPP (0.001 M), 0.6 unit of O5P decarboxylase (0.05 ml. of the ethanol fraction in the inorganic pyrophosphatase preparation; see "Materials"), and the enzyme preparation, in a volume of 1.0 ml. The rate of reaction was measured in a Beckman DU spectrophotometer. A unit of enzyme was defined as the amount causing the removal of 1 μmole of orotate in 1 hour, calculated from the initial reaction rate (4 to 8 minutes). The specific activity was defined as units of enzyme per mg. of protein.

Under the conditions of the assay, the rate of orotate removal was proportional to the amount of enzyme. Thus the decrease in optical density at 295 mμ in 8 minutes per ml. of enzyme was found to be 0.00, 0.56, 0.53, 0.49, and 0.46 with 0.00, 0.02, 0.04, 0.07, and 0.10 ml. of enzyme solution, respectively. In the absence of PRPP no decrease in density was observed.

Assay of O5P Decarboxylase—The conversion of O5P to U5P (equation (2)) was measured by the decrease in optical density at 285 mμ. The test system (1.0 ml.) contained 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), 0.03 to 0.05 μmole of O5P, and the enzyme preparation. A unit of enzyme was defined as the amount causing decarboxylation of 1 μmole of O5P ($-\Delta = 1.65$) in 1 hour, calculated from the initial reaction rate (0 to 4 minutes).

The decarboxylation of 4,7-C^{14}-O5P was measured in a Thunberg tube

by trapping the $C^{14}O_2$ in NaOH contained in the side bulb, plating the Na_2CO_3 on aluminum disks, and estimating the radioactivity in a gas flow counter.

Synthesis of O5P (by Phosphate Transfer Reaction) and Its Identification—O5P was synthesized by a phosphate transfer from phenyl phosphate to orotidine with a phosphatase obtained from commercial malt diastase (13). The phosphatase preparation (0.4 ml.) (see "Materials") was incubated at 36° with 2.0 ml. of disodium phenyl phosphate (0.082 M, dissolved in 0.2 M sodium acetate buffer and adjusted to pH 5.0) and 47 mg. of orotidine in a final volume of 4.0 ml. After 16 hours, the reaction mixture was adjusted to pH 7.0 and chromatographed on a column of Dowex 1, chloride form (2 per cent cross-linked, height 6 cm., diameter 2.2 cm.). Orotidine was eluted with 0.01 N HCl (peak at 31.1 resin bed volumes of eluent), phenyl phosphate with a solution of 0.005 N HCl-0.02 M KCl (peak at 10.0 resin bed volumes of this eluent), and O5P with a solution of 0.01 N HCl-0.1 M KCl (eluted between 8.0 and 14.9 resin bed volumes of this eluent; peak at 10.0 volumes).

O5P (7.7 μmoles) was characterized on the basis of its absorption spectrum (identical with that of orotidine (12); peak at 266 mμ, λ280/λ260 = 0.65 at pH 7.0), by its enzymatic decarboxylation to U5P (equation (2)) (also see below), and by the molar ratios of orotic acid (after hydrolysis for 3 hours at 100° in 2.5 N H_2SO_4) to pentose to phosphate[5] of 1.08:1.04:1.06.

Results

Purification of O5P Pyrophosphorylase

O5P pyrophosphorylase was purified about 20-fold from extracts of yeast (Table I). The operations were carried out at 0° unless otherwise indicated.

Preparation of Autolysate—Dried brewers' yeast was suspended in 3 volumes of $KHCO_3$ (0.1 M) and incubated at 30° for 5 hours with occasional stirring. After cooling the suspension to 5–10°, the supernatant fluid was obtained by centrifugation in a Servall type SS-1 centrifuge at about 10,000 × *g*. The residue was washed with an amount of $KHCO_3$ solution equal to that added originally and after centrifugation the supernatant fluids were combined. For 10 gm. of dried yeast the yield of extract was 45 to 50 ml.

Ethanol Fraction—To 48 ml. of yeast extract 96 ml. of sodium acetate buffer (0.2 M, pH 4.5) were added rapidly with stirring. The acidified

[5] No detectable phosphate was released by 5′-nucleotidase during the time required for complete hydrolysis of an equivalent amount of U5P. The presence of O5P did not inhibit the rate of the enzymatic hydrolysis of U5P.

extract was cooled to −2° in an alcohol-ice bath, and 48 ml. of ethanol (−14°) were added (in approximately 4 minutes) while the temperature was allowed to fall to −6°. Insoluble material was removed by centrifugation for 4 minutes at 10,000 × *g* in a Servall centrifuge kept at −14°. 144 ml. of ethanol were then added to the supernatant solution (in approximately 5 minutes) with the temperature maintained at −10°. The precipitate, collected by centrifugation (3 minutes, 10,000 × *g*), was dissolved in glycylglycine buffer (0.025 M, pH 7.0) and the pH was adjusted to 7.0 with 1 N KOH. Volume = 48 ml. (ethanol fraction).

Calcium Phosphate Gel Adsorption—To the ethanol fraction (48 ml.) was added sufficient calcium phosphate gel (17) (170 mg. of solids) to adsorb 85 to 90 per cent of the O5P pyrophosphorylase activity. After 5 minutes

TABLE I

Purification of O5P Pyrophosphorylase

Total units of O5P decarboxylase	Enzyme fraction	Total units of O5P pyrophosphorylase	Over-all recovery	Specific activity
			per cent	*units per mg. protein*
274	Autolysate	532		0.7
240	Ethanol fraction	240	45.1	1.7
2.9	Calcium phosphate gel fraction	141	26.5	6.7
0.0	Aluminum hydroxide gel fraction	83	15.6	17.3

the mixture was centrifuged and the supernatant fluid was discarded. The enzyme was eluted from the precipitate with 24 ml. of Tris buffer (0.2 M, pH 8.0) (calcium phosphate gel fraction).

Aluminum Hydroxide Gel Adsorption—The calcium phosphate gel fraction (24 ml.) was adjusted to pH 7.0 with 1 N acetic acid and 24 ml. of water were added. Sufficient aluminum hydroxide gel Cγ (18) (29 mg. of solids) was then added to adsorb all the O5P pyrophosphorylase. The precipitate was collected after 5 minutes and washed with 48 ml. of potassium phosphate buffer (0.01 M, pH 7.2). The enzyme was then eluted with 48 ml. of potassium phosphate buffer (0.06 M, pH 7.2) (aluminum hydroxide gel fraction).

O5P Pyrophosphorylase in Animal Tissues—This activity appears to be present in extracts of liver acetone powders prepared from several animal species. The release of $C^{14}O_2$ from 4,7-C^{14}-orotate was used as an assay,[6]

[6] 100 mg. of liver acetone powder (6) were extracted for 10 minutes at 0° with 1 ml. of 0.02 M Tris buffer, pH 8.0. The reaction mixture in Thunberg tubes contained, in

since the order of activity of the crude extracts was too low to permit spectrophotometric measurements. The specific activities of the extracts from pigeon, beef, chicken, hog, and lamb were 0.022, 0.020, 0.018, 0.011, and 0.004, respectively.

Stoichiometry, Equilibrium, and Properties of O5P Pyrophosphorylase Reaction

Stoichiometry of Condensation of Orotate and PRPP—The stoichiometry of the over-all reaction starting with orotate and PRPP (equations (1) and (2)) was studied with the ethanol fraction (Table I) which contained both O5P pyrophosphorylase and O5P decarboxylase (Table II). Good agreement was observed between the disappearance of orotate and PRPP on the one hand and the formation of U5P, CO_2, and PP on the other.

Accumulation of O5P—With a more purified enzyme fraction (aluminum hydroxide gel fraction) which was free of the decarboxylase, the synthesis of O5P from orotate and PRPP was demonstrable. The reaction mixture (18.0 ml.) contained 360 μmoles of Tris buffer (pH 8.0), 36 μmoles of $MgCl_2$, 0.93 μmole of PRPP, 0.43 μmole of 4,7-C^{14}-sodium orotate (94,250 c.p.m. per μmole), and 3.4 units of O5P pyrophosphorylase (specific activity 15). After incubation at 30° for 15 minutes, 0.27 μmole of orotate had been consumed as indicated by the decrease in optical density at 295 mμ. The reaction mixture was heated for 2 minutes in boiling water and then chromatographed at 3° on a column of Dowex 1, chloride form (2 per cent cross-linked, height 3 cm., diameter 1 cm.). Orotic acid was eluted with a solution of 0.0025 N HCl-0.1 M KCl and O5P with a solution of 0.01 N HCl-0.1 M KCl. 0.26 μmole of O5P was recovered. It was identified by its absorption spectrum (peak at 266 mμ, λ280/λ260 = 0.66 at pH 7.0) and by enzymatic decarboxylation (0.26 μmole of $C^{14}O_2$ liberated) (see equation (2)).

The synthesis of larger amounts of O5P proved difficult, owing to an early decline in reaction rate which occurred with the purified enzyme preparation. The decreased rate appears to be due to the inhibitory action of O5P, since (1) a linear rate of orotate removal was observed when an O5P decarboxylase preparation was added and (2) the addition of O5P caused a marked inhibition of the reaction (Fig. 1). The addition of inorganic pyrophosphatase to augment that present in the O5P pyrophosphorylase had no effect on the rate of orotate utilization in the presence or absence of added O5P.

1.0 ml., 20 μmoles of Tris buffer (pH 8.0), 5 μmoles of $MgCl_2$, 0.26 μmole of PRPP, 0.5 μmole of 4,7-C^{14}-orotate (140,000 c.p.m. per μmole), 0.1 unit of O5P decarboxylase, and 0.2 ml. of the liver extract. After incubation for 30 minutes at 30°, acid was admitted to the tubes and the released $C^{14}O_2$ was trapped and counted.

Stoichiometry of Pyrophosphorolysis of O5P—To study the stoichiometry of the O5P pyrophosphorylase reaction in the direction of orotate formation (equation (1)), a reaction mixture was prepared containing the purified enzyme, O5P, and PP^{32}. The extent of O5P utilization was matched by the removal of PP and the appearance of orotate and PRPP (Table III).

TABLE II

Stoichiometry of Condensation of Orotate and PRPP

The reaction mixture (24 ml.) contained 0.48 ml. of Tris buffer (1 M, pH 8.0), 0.48 ml. of $MgCl_2$ (0.1 M), 1.2 ml. of PP (0.01 M, pH 8.5), 1.44 ml. of NaF (0.5 M), 0.48 ml. of 4,7-C^{14}-sodium orotate (0.01 M, 186,000 c.p.m. per μmole), 0.40 ml. of P^{32}-labeled PRPP (2.85×10^{-3} M, 112,000 c.p.m. per μmole), and 15.9 units of O5P pyrophosphorylase (ethanol fraction, specific activity 1.3). 12 ml. of this reaction mixture were placed in a boiling water bath for 0.5 minute at zero time; the remainder was incubated at 30° for 40 minutes and then heated in a boiling water bath for 0.5 minute. Orotate was measured spectrophotometrically at 295 mμ. U5P was determined spectrophotometrically by the increase in optical density at 260 mμ. $C^{14}O_2$ release was estimated with 1 ml. reaction mixtures in Thunberg tubes. The values in bold-faced type were determined after anion exchange chromatography of aliquots of the reaction mixtures. Orotic acid and U5P were separated from each other on Dowex 1, formate form (10 per cent cross-linked, eluent = 0.1 M sodium formate adjusted to pH 3.2 with formic acid), and estimated at 280 and 262 mμ, respectively. PRPP and PP were separated from each other on Dowex 1, chloride form (2 per cent cross-linked, eluent = 0.0025 N HCl-0.1 M KCl), and estimated by radioactivity measurements. All values are expressed in micromoles.

	0 min.	40 min.	Δ
Orotate	2.40	1.84	−0.56
"	**2.38**	**1.87**	**−0.51**
PRPP	0.57	0.03	−0.54
"	**0.52**	**0.00**	**−0.52**
U5P	0.00	0.55	+0.55
"	**0.00**	**0.55**	**+0.55**
CO_2	0.00	0.60	+0.60
PP	**0.09**	**0.48**	**+0.39***

* This value is low because of inorganic pyrophosphatase activity; 0.22 μmole was recovered as 0.44 μmole of inorganic orthophosphate.

Determination of Equilibrium Constant for O5P Pyrophosphorylase Reaction—The equilibrium of the reversible pyrophosphorolysis of O5P was studied spectrophotometrically, in both directions, with the purified enzyme (Fig. 2). In addition to the substrates, one or both products of the pyrophosphorylase reaction were added initially to allow equilibrium to be reached in a shorter time. An equilibrium constant, calculated (in the direction of O5P synthesis) at each of the five plateau areas, yielded values of 0.17, 0.11, 0.10, 0.13, and 0.11, with an average value of 0.12.

Effect of Substrate Concentration—The rate of the O5P pyrophosphorylase reaction in the presence of the decarboxylase was studied as a function of orotate and PRPP concentration (Fig. 3). Lineweaver and Burk plots (19) yielded straight lines. The *Ks* values were found to be the same for both substrates, 2.0×10^{-5} M. To study the effect of PP concentration on the rate of pyrophosphorolysis, a similar experiment was carried out

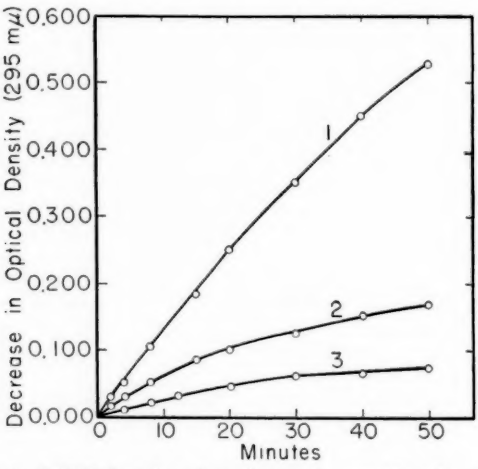


FIG. 1. Effect of O5P decarboxylase, inorganic pyrophosphatase, and O5P on the rate of the O5P pyrophosphorylase reaction. The reaction mixtures (1.0 ml.) contained Tris buffer, $MgCl_2$, orotate, and PRPP as for the standard assay (see the text), and 0.13 unit of O5P pyrophosphorylase (specific activity 17.4) free of decarboxylase. Further additions were as follows: Curve 1, 0.6 unit of O5P decarboxylase, 820 units of impure inorganic pyrophosphatase (see "Materials"). Curve 2, no additions *or* 3340 units of crystalline inorganic pyrophosphatase. Curve 3, 0.031 μmole of O5P with or without 3340 units of crystalline inorganic pyrophosphatase. Utilization of orotate was followed by the decrease in optical density at 295 mμ.

in the presence of a constant amount of O5P (Fig. 4). *Ks* was calculated to be 1.2×10^{-4} M.

Effect of pH and Mg^{++} on O5P Pyrophosphorylase Reaction—The rate of O5P pyrophosphorolysis was studied as a function of pH with the purified enzyme and Tris-maleate buffers. Maximal rates (measured spectrophotometrically during a 10 minute interval) were obtained in the range 6.0 to 7.0. At pH 5.5, 8.0, and 8.6, the reaction rates were 79, 77, and 57 per cent, respectively, of the maximal rate. The optimal pH range for the condensation of orotate and PRPP was from 7.5 to 8.5.

In the absence of added Mg^{++}, the rate of O5P formation by the purified

enzyme was decreased to about 25 per cent of that observed with 0.002 M Mg^{++}.

Specificity of O5P Pyrophosphorylase—No utilization of PRPP was detected when adenine, uracil, cytosine, DL-ureidosuccinate, L-dihydroorotate, or orotidine was used in place of orotate. Nor did the O5P pyrophos-

TABLE III

Stoichiometry of Pyrophosphorolysis of O5P

The reaction mixture (21.6 ml.) contained 0.36 ml. of Tris buffer (1 M, pH 8.0), 0.36 ml. of $MgCl_2$ (0.1 M), 0.8 ml. of NaF (0.05 M), 1.8 ml. of PP (1.2×10^{-3} M, 750,000 c.p.m. per μmole), 13.2 ml. of O5P (9.8×10^{-5} M), and 58.7 units of O5P pyrophosphorylase (specific activity 14.0). One-half of the reaction mixture was placed in a boiling water bath for 0.5 minute at zero time; the other half was incubated at 30° for 22 minutes and then placed in a boiling water bath for 0.5 minute. The disappearance of O5P was measured spectrophotometrically by the decrease in optical density at 266 mμ and the formation of orotate by the increase in optical density at 295 mμ. The values in bold-faced type were determined after chromatography of aliquots of the reaction mixtures on Dowex 1, chloride form (2 per cent cross-linked, height 3 cm., diameter 1 cm., eluent = 0.0025 N HCl-0.1 M KCl), at 3°. Orotic acid and O5P were estimated spectrophotometrically at 280 and 266 mμ, respectively; PP and PRPP by radioactivity measurements. All values are expressed in micromoles.

	0 min.	22 min.	Δ
O5P	0.65	0.39	−0.26
"	**0.58**	**0.29**	**−0.29**
PP	**1.04**	**0.84**	**−0.20***
Orotate	0.00	0.32†	+0.32
"	**0.00**	**0.30**	**+0.30**
PRPP	0.00	0.22	+0.22
"	**0.00**	**0.16**	**+0.16***

* The relatively low value for PRPP and the high value for PP at 22 minutes suggest the destruction of PRPP to yield PP.

† A value of 0.31 μmole was obtained for orotate production when determined with the dihydroorotic dehydrogenase system (7).

phorylase preparation convert any detectable amounts of adenine or uracil to their respective nucleotides when 8-C^{14}-adenine and 2-C^{14}-uracil of high specific activities were used. When ribose-1,5-diphosphate was used in place of PRPP, no removal of orotate could be observed. Inorganic triphosphate was inactive in place of PP.

Decarboxylation of O5P to Form U5P

Identification of U5P As Product of Decarboxylation of O5P—The action of O5P decarboxylase on O5P yielded a compound with the absorption

spectrum of U5P (Fig. 5). Larger amounts of U5P, for purposes of additional characterization, were obtained from a reaction mixture containing (in 10.0 ml.) 5 μmoles of 4,7-C^{14}-orotate (51,000 c.p.m. per μmole), 5.3

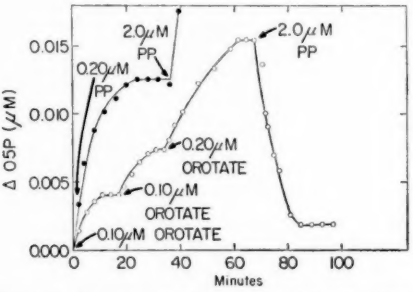


FIG. 2. Determination of the equilibrium constant for the O5P pyrophosphorylase reaction. The reaction mixtures (1.0 ml.) contained 20 μmoles of Tris buffer (pH 8.0), 2 μmoles of $MgCl_2$, 0.1 μmole of PRPP, 5 μmoles of NaF, and 0.17 unit of O5P pyrophosphorylase (specific activity 17.4). ●, course of O5P pyrophosphorolysis (−Δ O5P); 0.023 μmole of O5P, 0.1 μmole of orotate, and the indicated amounts of PP were added. ○, course of O5P formation (+Δ O5P); 0.25 μmole of PP and the indicated amounts of orotate were added. The progress of the reactions was followed by measuring the increase or decrease in optical density at 295 mμ.

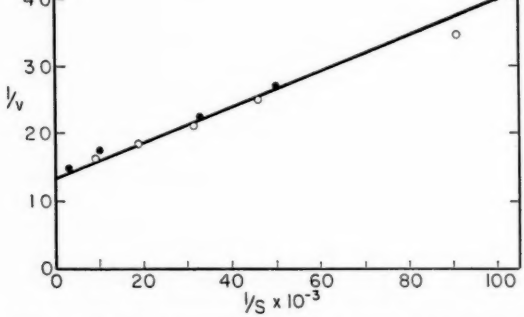


FIG. 3. The rate of the O5P pyrophosphorylase reaction as a function of orotate and PRPP concentration. The reaction mixtures containing 0.17 unit of O5P pyrophosphorylase (specific activity 17.3) were prepared as for the standard assay (see the text), except that varying amounts of sodium orotate or PRPP (molar concentrations = *S*) were used. The rate of orotate utilization was measured by the decrease in optical density at 295 mμ during a 10 minute interval (*v*). ○, a Lineweaver and Burk (19) plot of the results obtained with varying amounts of orotate; ●, with varying amounts of PRPP.

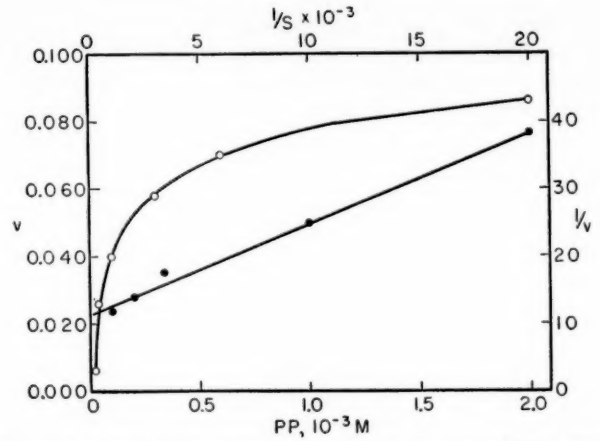


FIG. 4. The rate of O5P pyrophosphorolysis as a function of PP concentration. The reaction mixtures (1.0 ml.) contained 20 μmoles of Tris buffer (pH 8.0), 2 μmoles of $MgCl_2$, 0.039 μmole of O5P, 0.17 unit of O5P pyrophosphorylase (specific activity 17.3), and the indicated amounts of PP (molar concentrations = S). The progress of the reaction was followed by measuring the increase in optical density at 295 mμ during a 10 minute interval (v). ○, increase in optical density as a function of PP concentration; ●, a Lineweaver and Burk plot (19).

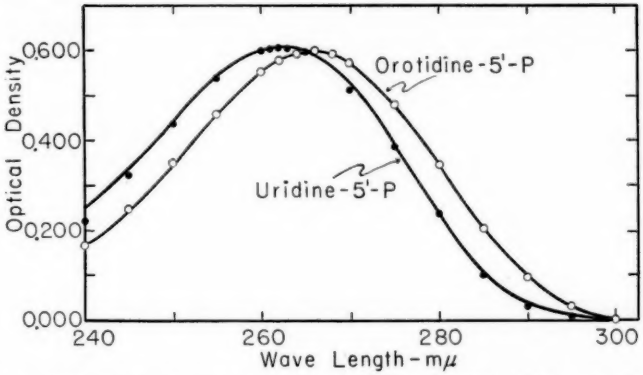


FIG. 5. U5P, the product of the decarboxylation of O5P. The incubation mixture (1.0 ml.) in the experimental cuvette contained 20 μmoles of Tris buffer (pH 8.0), 2 μmoles of $MgCl_2$, 0.05 μmole of O5P (prepared with malt phosphatase), and 0.73 unit of O5P decarboxylase (ethanol fraction, Table I). The reaction mixture in the blank cell contained no O5P. The course of the reaction was followed by the decrease in optical density at 285 mμ. When the reaction was complete (after 10 minutes), the absorption spectrum of the product was determined. The curves represent the spectra of O5P and authentic U5P, as indicated; ● represents the final values obtained in the incubation mixture.

μmoles of PRPP, and an enzyme preparationi wth both O5P pyrophosphorylase and decarboxylase activities (ethanol fraction, Table I). When 3.43 μmoles of orotate had been utilized, the reaction was stopped and the product was isolated by ion exchange chromatography. 3.04 μmoles were recovered and identified as U5P on the basis of (1) absorption spectrum ($\lambda 280/\lambda 260 = 0.36$ at pH 3.2), (2) properties on ion exchange chromatography (Dowex 1, formate form, 10 per cent cross-linked, eluted between 8 and 18 resin bed volumes of 0.1 M sodium formate, pH 3.2, with peak at 12 volumes), and (3) by the release of 2.86 μmoles of phosphate by 5′-nucleotidase (14). The specific activity of the U5P (24,700 c.p.m. per μmole) was almost exactly one-half of the 4,7-C^{14}-orotate.

Irreversibility of O5P Decarboxylation—To determine whether O5P can be synthesized from U5P and CO_2, a reaction mixture containing U5P and $NaHC^{14}O_3$ (10^6 c.p.m. per μmole) was incubated. No detectable radioactivity was retained in the reaction mixture after acidification.

DISCUSSION

The definition of a pathway of conversion of orotic acid to uridine nucleotide provides a clearer basis for investigating a number of unexplained observations. The fact that some microorganisms utilize only uracil while others (and some animal tissues too) utilize only orotic acid (20) can be ascribed to different metabolic pathways or to differences in cell permeability. In either case, a determination of the concentration and the characteristics of the O5P pyrophosphorylase and decarboxylase in these cells is essential. Similar considerations apply to the genetic defect in pyrimidineless mutants. The accumulation of orotic acid (21) and orotidine (12) by certain mutants of *Neurospora* may be due to a relative lack of O5P decarboxylase. Thus, piling up of O5P might, as in the case of yeast O5P pyrophosphorylase, inhibit orotic acid utilization and the action of phosphatases on O5P would then lead to the formation of orotidine.

SUMMARY

1. The conversion of orotic acid to uridine-5′-phosphate (U5P) has been studied with enzymes partially purified from yeast and found to proceed according to the following equations:

Orotic acid + 5′-phosphoribosylpyrophosphate (PRPP) ⇄
orotidine-5′-phosphate (O5P) + inorganic pyrophosphate

Orotidine-5′-phosphate → uridine-5′-phosphate + CO_2

O5P pyrophosphorylase activity appears to be present also in the liver of several animal species.

2. The formation of O5P and the pyrophosphorolysis of O5P proceed readily to equilibrium. The constant in the direction of O5P synthesis has been calculated to be approximately 0.1. Reversibility of O5P decarboxylation was not demonstrable.

3. O5P synthesized by phosphate transfer from phenyl phosphate to orotidine by a malt phosphatase appears to be identical in its spectral and chromatographic properties and enzyme reactivity to that obtained by the enzymatic condensation of orotate and PRPP.

4. The yeast O5P pyrophosphorylase was specific for orotic acid among a number of pyrimidines and pyrimidine precursors tested. No reaction was observed when PRPP was replaced by ribose diphosphate.

BIBLIOGRAPHY

1. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L., and Wilson, D. W., *J. Am. Chem. Soc.*, **73,** 1898 (1951).
2. Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., von Ubisch, H., and Bergström, S., *J. Biol. Chem.*, **179,** 169 (1949).
3. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195,** 257 (1952).
4. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2027 (1954).
5. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2844 (1954).
6. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 389 (1955).
7. Lieberman, I., and Kornberg, A., *Biochim. et biophys. acta*, **12,** 223 (1953); *J. Biol. Chem.*, **207,** 911 (1954); **212,** 909 (1955).
8. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 417 (1955).
9. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 429 (1955).
10. Klenow, H., *Arch. Biochem. and Biophys.*, **46,** 186 (1953).
11. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
12. Michelson, A. M., Drell, W., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **37,** 396 (1951).
13. Brawerman, G., and Chargaff, E., *J. Am. Chem. Soc.*, **75,** 2020, 4113 (1953).
14. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **192,** 87 (1951).
15. Kunitz, M., *J. Gen. Physiol.*, **35,** 423 (1951–52).
16. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **188,** 665 (1951).
17. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124,** 397 (1938).
18. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56,** 1117 (1923).
19. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
20. Wright, L. D., Driscoll, C. A., Miller, C. S., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **84,** 716 (1953).
21. Mitchell, H. K., Houlahan, M. B., and Nyc, J. F., *J. Biol. Chem.*, **172,** 525 (1948).

ENZYMATIC SYNTHESIS OF PURINE NUCLEOTIDES*

By ARTHUR KORNBERG, IRVING LIEBERMAN, AND ERNEST S. SIMMS

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, November 22, 1954)

The demonstration of a pathway of pyrimidine nucleotide synthesis in which PRPP[1] is a key intermediate (1, 2) prompted a study to determine whether the same type of reaction mechanism might apply to the synthesis of purine nucleotides. In a preliminary note the condensation of adenine with PRPP to yield A5P was reported (3). Subsequent work has established the existence of enzymes in yeast which react with other purines (hypoxanthine and guanine) and supports a more generalized equation (1).

$$\text{Purine} + \text{PRPP} \rightleftarrows \text{purine nucleoside-5'-phosphate} + \text{PP}$$

This report describes the purification of these enzymes, their properties, and the stoichiometry of the reversible pyrophosphorolyses which they catalyze.

Methods

Materials—The unlabeled purines and orotic acid were products of Dougherty Chemicals. I5P and A5P were obtained from the Sigma Chemical Company. Hypoxanthine-8-C^{14}, guanine-8-C^{14} sulfate, and 2,6-diaminopurine-2-C^{14} sulfate were gifts from Dr. R. Abrams, Dr. H. A. Barker, and Dr. G. B. Brown, respectively. Orotic acid-2-C^{14} (4) and adenine-8-C^{14} sulfate (5) were prepared and kindly furnished by S. R. Kornberg. PRPP, P^{32}-labeled PRPP, and ribose-1,5-diphosphate were prepared as previously described (1, 2). Crystalline inorganic pyrophosphatase (6) was kindly furnished by Dr. G. Perlmann and Dr. M. Kunitz.

Determinations—Hypoxanthine was determined spectrophotometrically with xanthine oxidase (7).[2] Procedures used for PRPP, A5P, protein, and phosphate determinations, ion exchange chromatography, and radioactivity measurements were described previously (1).

* This investigation was supported by grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

[1] The abbreviations used are 5-phosphoribosylpyrophosphate, PRPP; adenosine-5'-phosphate, A5P; inosine-5'-phosphate, I5P; tris(hydroxymethyl)aminomethane, Tris; inorganic pyrophosphate, PP; guanosine-5'-phosphate, G5P; ribose-1-phosphate, R1P; inorganic orthophosphate, P.

[2] Kindly furnished by Dr. M. Friedkin.

XUM

Adenine was separated from A5P (but not from adenosine) in an incubation mixture by a chromatographic procedure that can be carried out in about 10 minutes. The mixture was adjusted to about pH 6.5 with brom thymol blue as an internal indicator and adsorbed on a column (2.5 cm. × 1 sq. cm.) of Dowex 1, chloride form (2 per cent cross-linked, 200 to 400 mesh), contained in a sintered glass (very coarse) funnel about 5 cm. tall. The column, set in a suction flask, was washed with 2.5 ml. of water and then with 5.0 ml. of 0.002 N HCl. The HCl eluate contained nearly 100 per cent of the adenine as determined by optical density at 260 mμ or by radioactivity measurements; no trace of A5P was present in this eluate. The use of a battery of such small columns attached to a manifold under hydrostatic pressure of wash or eluting fluids will be described in the succeeding paper (8).

Enzyme Assays—The rate of A5P synthesis was routinely determined by the spectrophotometric estimation of A5P with deaminase (Assay A). With some crude tissue extracts, assays were based on the incorporation of C^{14}-adenine into an A5P fraction (Assay B).

Assay A. A5P Synthesis Determined Spectrophotometrically—The incubation mixture (1.0 ml.) contained 0.1 ml. of adenine (1.4×10^{-3} M), 0.1 ml. of PRPP (0.001 M), 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), and less than 0.2 unit of enzyme. The mixture was incubated at 36° for 15 minutes, then placed in a boiling water bath for 1 minute and centrifuged to remove any precipitate which formed on heating. 0.5 ml. of the supernatant fluid was analyzed for A5P by adding it to 0.5 ml. of succinate buffer (0.2 M, pH 6.0) in a cuvette and, after an initial reading, adding 0.02 ml. of a deaminase preparation. The molar absorption coefficient for the density decrease at 265 mμ was taken to be 8700. 1 unit of enzyme is defined as that amount causing the formation of 1 μmole of A5P (as indicated by density decrease on deamination or by the incorporation of 1 μmole of C^{14}-adenine (Assay B, below)) per hour and specific activity as units per mg. of protein.

Assay B. Incorporation of C^{14}-Adenine into Insoluble Barium Compound—The incubation mixture (0.4 ml.) contained 0.03 ml. of C^{14}-adenine (0.01 M, 1×10^5 c.p.m. per μmole), 0.1 ml. of PRPP (0.001 M), 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), and less than 0.2 unit of enzyme. The mixture was incubated at 36° for 15 minutes and then treated with 0.15 ml. of 10 per cent trichloroacetic acid. The precipitate was removed by centrifugation and to the supernatant fluid were added 0.1 ml. of A5P (0.025 M) as carrier, 1 drop of thymol blue (0.04 per cent), 2 drops of 2 N KOH, 0.1 ml. of saturated barium acetate, and 2.4 ml. of ethanol. The precipitate was washed twice with 3 ml. portions of ethanol and suspended in 0.5 ml. of 0.1 N HCl. An aliquot (0.1 to 0.2 ml.) of the

suspension was plated on a round glass cover-slip for radioactivity measurement and corrections for self-absorption were applied.

Results

Adenosine-5'-phosphate

Purification of A5P Pyrophosphorylase—All operations were carried out at 0–3°. An autolysate of dried brewers' yeast was prepared as previously described (2) (Table I).

To 20 ml. of autolysate were added, with stirring, 40 ml. of potassium acetate buffer (0.2 M, pH 4.5). The mixture was cooled to 0° in a −15° alcohol-ice bath and then treated with 11.5 ml. of −15° ethanol. The precipitate was removed by centrifugation and to the supernatant fluid (now −6°) was added more cold ethanol (15.0 ml.) under constant stirring.

TABLE I

Purification of A5P Pyrophosphorylase

Step	Units per ml.	Total units	Protein	Specific activity
			mg. per ml.	*units per mg. protein*
1. Autolysate	18.8	376	15.6	1.2
2. Ethanol	4.7	188	2.1	2.2
3. Aluminum hydroxide gel eluate	2.8	108	0.45	6.2
4. Calcium phosphate gel supernatant	1.2	72	0.05	24.0

The temperature was −10°. The precipitate was collected by centrifugation and dissolved in 40 ml. of glycylglycine buffer (0.025 M, pH 6.8), and the pH was adjusted to about 6.8 with KOH (about 4 drops of 0.1 N required) (ethanol fraction).

The ethanol fraction (40 ml.) was treated with 2.0 ml. of aluminum hydroxide gel Cγ (9) (15 mg. of solids per ml.). After 5 minutes at 0°, the gel was collected by centrifugation and washed with 40 ml. of sodium acetate buffer (0.1 M, pH 5.95), and then the enzyme was eluted with 40 ml. of potassium phosphate buffer (0.01 M, pH 7.2) (aluminum hydroxide gel eluate).

The aluminum hydroxide gel eluate (40 ml.) was diluted to 48 ml. with water and then 12 ml. of calcium phosphate gel (10) (17 mg. of solids per ml.) were added slowly with stirring. After 5 minutes at 0°, the gel was removed by centrifugation (calcium phosphate gel supernatant).

The ethanol and gel-treated fractions retained from 65 to 75 per cent of their activity after 6 weeks storage at −15°.

Distribution of Enzyme—As determined by Assay B, the conversion

of adenine to A5P is catalyzed by the extracts of liver of several animal species. Extracts of liver acetone powders (1) of the lamb, hog, pigeon, chicken, rat, and cow were all active, with specific activities descending in that order. On a protein basis, extract from lamb liver was about 6 per cent as active as yeast autolysate, while that from beef liver was only 2 per cent as active.

Balance Study of A5P Synthesis—The stoichiometry of the condensation of adenine with PRPP to yield A5P and PP is shown in Table II. Values

TABLE II

Balance Study of A5P Synthesis

The incubation mixture (10.0 ml.) contained 4.0 ml. of adenine (0.0016 M), 0.5 ml. of $PRP^{32}P^{32}$ (0.0028 M, 1.2×10^5 c.p.m. per μmole), 0.2 ml. of NaF (0.05 M), 0.2 ml. of Tris buffer (1 M, pH 8.0), 0.2 ml. of $MgCl_2$ (0.1 M), and 3.0 ml. of the calcium phosphate gel eluate fraction. The mixture was incubated at 36° for 60 minutes and then placed in a boiling water bath for 1 minute. The 0 minute sample was heated prior to the addition of the $PRP^{32}P^{32}$. The values are expressed in micromoles. The values in bold-faced type were obtained by chromatography of 8.0 ml. of the reaction mixtures on Dowex 1 columns, chloride form (3 cm. × 1 sq. cm.); the eluent was 0.0025 N HCl for 10 resin bed volumes and then was replaced by 0.0025 N HCl-0.1 M KCl. The other values were obtained by direct enzymatic assay of aliquots of the incubation mixtures.

	0 min.	60 min.	Δ
Adenine	**6.40**	**5.25**	**−1.15**
PRPP	1.22	0.00	−1.22
"	**1.19**	**0.01**	**−1.18**
A5P	0.00	1.27	+1.27
"	**0.00**	**1.33**	**+1.33**
PP	**0.16**	**1.10**	**+0.94***

* 0.34 μmole of inorganic orthophosphate was also produced.

obtained by direct enzymatic assay and by ion exchange chromatographic analysis were in good agreement.

In the presence of an excess of adenine, the PRPP was quantitatively converted to A5P. Similarly with an excess of PRPP over adenine, A5P formation was a quantitative measure of the adenine added (Table III).

This reaction has been utilized for the synthesis (and isolation) of C^{14}-labeled A5P produced from C^{14}-labeled adenine, thereby providing a useful reagent for studies of the reversal of the reaction. A 50 ml. incubation mixture contained adenine (28 μmoles, 7.5×10^4 c.p.m. per μmole), PRPP (34 μmoles), Tris buffer (0.02 M, pH 8.0), $MgCl_2$ (0.002 M), and the enzyme (20 ml. of the ethanol fraction or 15 ml. of the alumina gel eluate fraction). After an incubation period of 75 minutes at 36°, 23 μmoles of A5P were

formed. The mixture was heated in a boiling water bath for 2 minutes and then chromatographed on a Dowex 1 column (chloride form, 4 cm. × 2 sq. cm.). Adenine was eluted with 0.001 N HCl (complete after 40 resin bed volumes) and A5P was then promptly eluted with 0.05 N HCl. 20 μmoles of A5P were recovered of which 18.4 were in a single resin bed volume (8 ml.). Further concentration and desalting when desired were achieved by adsorption on Norit and elution with ammoniacal ethanol.

A5P Pyrophosphorolysis—Consistent with the above indications that the reaction favors nucleotide formation was the failure with ordinary methods to demonstrate A5P pyrophosphorolysis. However, by the use of C^{14}-labeled A5P or P^{32}-labeled PP, the formation of adenine and PRPP could

TABLE III

Quantitative Conversion of Adenine or PRPP to A5P

The incubation mixture in the "adenine excess" experiment contained (in 1.0 ml.) 0.2 ml. of adenine (0.001 M), additions of PRPP (0.00155 M) as indicated, 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), and 0.3 ml. of the enzyme (ethanol fraction). In the "PRPP excess" experiment, the incubation mixtures contained (in 1.0 ml.) 0.05 ml. of PRPP (0.003 M), additions of A5P (0.0014 M) as indicated, 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of $MgCl_2$ (0.1 M), and 0.1 ml. of the enzyme (aluminum hydroxide gel eluate fraction). The incubation was for 60 minutes at 36°. The reaction was stopped by immersing the sample in a boiling water bath for 1 minute. The values are expressed in micromoles.

	Adenine (added in excess)				PRPP (added in excess)			
Added adenine.....	0.20	0.20	0.20	0.20	0.028	0.056	0.084	0.112
" PRPP.....	0.023	0.046	0.092	0.155	0.15	0.15	0.15	0.15
Formed A5P.....	0.026	0.044	0.086	0.139	0.029	0.055	0.086	0.096

be demonstrated (Table IV). In this experiment (and in two similar ones) about 3.5 per cent of the A5P was converted to adenine. When the concentration of PP was reduced from 0.005 to 0.001 M or to 0.0002 M, the extent of A5P conversion, under otherwise similar conditions, fell to 1.1 and 0.6 per cent, respectively. In the absence of PP or enzyme, no adenine formation was detectable. That no adenosine was formed was indicated by the complete absence of radioactivity in the adenosine fraction of the chromatograms (Table IV).

Affinity and Specificity of Adenine and PRPP by A5P Pyrophosphorylase—Even at an adenine concentration of 4.5×10^{-5} M, the lowest level which could be tested with the more sensitive Assay B, there was no significant decrease in the reaction rate as compared with adenine levels 5 and 10 times as great. The dissociation constant of the adenine-enzyme complex must, therefore, be well below 4.5×10^{-5} M. The affinity of PRPP

for the enzyme also appeared to be great. With the smallest concentration tested, 4×10^{-5} M, the rate was about 60 per cent as rapid as at concentrations several times as great.

TABLE IV

Adenine and PRPP Production by A5P Pyrophosphorolysis

Experiment 1—The incubation mixture (5.00 ml.) contained 0.20 ml. of A5P (0.0022 M, 7.5×10^4 c.p.m. per μmole), 0.25 ml. of PP (0.1 M), 0.1 ml. of Tris buffer (1 M, pH 8.0), 0.1 ml. of $MgCl_2$ (0.1 M), 0.05 ml. of NaF (0.5 M), and 1.25 ml. of the calcium phosphate gel supernatant fraction. After incubation for 2 hours at 36°, the mixture was immersed in a boiling water bath for 2 minutes. Adenosine (0.04 μmole) and adenine (0.25 μmole) were added as carriers; the pH was adjusted to 11 and the volume to 6.0 ml. 5.5 ml. were adsorbed on a column of Dowex 1, chloride form (3 cm. × 1 sq. cm.), and eluted first with 0.01 M NH_4Cl-0.1 M NH_4OH for 3 resin bed volumes (sufficient to elute adenosine), and then with 0.01 M NH_4Cl. Each fraction represents about 1 resin bed volume.

Experiment 2—The incubation mixture was the same as that in Experiment 1, except that the A5P was unlabeled, the PP was labeled (2×10^6 c.p.m. per μmole), and phosphate (0.10 ml. of 1 M, pH 7.4) was added to stabilize the PRPP that was formed. After incubation for 2 hours at 36°, the following were then added: 0.4 ml. of PRPP (0.0055 M) as carrier, 3.6 ml. of water, and 1.0 ml. (containing about 0.5 mg.) of inorganic pyrophosphatase. After an incubation sufficient to destroy all the PP (20 minutes at 36°), the mixture was adsorbed on a column (3 cm. × 1 sq. cm.) of Dowex 1, chloride form, and eluted with 0.0025 N HCl-0.1 M KCl. Each fraction represents about 1 resin bed volume.

Experiment 1, isolation of adenine				Experiment 2, isolation of PRPP			
Fraction No.	Adenine	Radioactivity	Specific radioactivity	Fraction No.	PRPP	Radioactivity	Specific radioactivity
	μmole	*c.p.m.**	*c.p.m. per μmole*		*μmole*	*10^3 c.p.m.*	*c.p.m. per μmole*
6	0.000	0		23	0.020	1080	54,000
7	0.045	201	4470	25	0.136	4140	30,400
8	0.092	423	4590	27	0.189	5460	29,900
9	0.089	364	4080	29	0.138	3900	28,300
10	0.000	0		31	0.086	2580	29,900
				33	0.051	1680	32,900

* No other fractions in this chromatogram contained radioactivity. The recovery of carrier adenosine and adenine, as judged by optical density at 260 mμ, was about 90 per cent.

When adenine was replaced by hypoxanthine or guanine, the respective nucleotides of these purines were formed. A separate enzyme appears to be responsible for inosinic acid synthesis (see below); the nature of the "guanine enzyme" has not been determined. 2,6-Diaminopurine was inert as a substrate with the aluminum hydroxide gel eluate enzyme fraction. The

labeled purine (0.5 μmole, 3.7×10^5 c.p.m. per μmole) was incubated under conditions identical to those to be described below for guanine. No PRPP was consumed and on the basis of radioactivity in the nucleotide zone of an anion exchange chromatogram, there was no evidence for the formation of a nucleotide. Substitution of PRPP by ribose-1,5-diphosphate (presumably at a concentration of 4×10^{-5} M) resulted in no detectable A5P synthesis from adenine (<5 per cent of that obtained with PRPP).

Influence of pH, Mg^{++}, Fluoride, and PP on Rate—The optimal pH was around 7.6. The rates at pH 6.5 and 8.4 were 67 per cent of the maximal value. Without added Mg^{++}, the rate was 7 per cent or less of that obtained with Mg^{++} added to attain levels of 2×10^{-3} M or higher. The rate was half maximal at an Mg^{++} concentration of 2×10^{-4} M. Fluoride (0.05 M) inhibited the rate by 25 to 40 per cent. PP inhibited the rate by about 50 to 90 per cent at concentrations of 5×10^{-3} and 1×10^{-2} M, respectively. Elevation of the Mg^{++} level to 5×10^{-3} M failed to alter this inhibition by PP. Owing to the presence of inorganic pyrophosphatase, the influence of lower concentrations of PP was tested in the presence of fluoride (0.05 M). At levels of 1×10^{-3} and 2×10^{-3} M, the rates, corrected for fluoride inhibition, were 30 and 40 per cent inhibited, respectively.

Inosine-5'-phosphate

Nature of I5P Pyrophosphorylase—While the ethanol and aluminum hydroxide gel eluate fractions were active in the interconversion of hypoxanthine and I5P, treatment with calcium phosphate gel resulted in a preferential adsorption of this activity. The ratio of the rate of nucleotide synthesis with adenine as substrate to that with hypoxanthine as substrate in the unadsorbed fraction increased from approximately 2 to 20 (Table V).

Balance Study of I5P Synthesis—The removal of hypoxanthine and PRPP in approximately equal amounts was matched by the appearance of an equivalent quantity of I5P (Table VI), identified both by its chromatographic behavior and absorption spectrum.

I5P Pyrophosphorolysis—As in the case of A5P pyrophosphorolysis, the cleavage of I5P was slow and appeared to be limited. Under conditions comparable to those for A5P splitting (see Table IV), except that the concentrations of I5P and PP were 1×10^{-3} M, the hypoxanthine formed per ml. of reaction mixture in 1 hour was 0.0023 μmole. When PP was omitted or replaced by orthophosphate (1.5×10^{-2} M) or when I5P was omitted, no hypoxanthine formation was detectable. The extent of reaction increased with time and enzyme content and decreased with lower concentrations of PP and I5P. However, no kinetic or equilibrium data have been obtained.

Guanosine-5'-phosphate

The aluminum hydroxide gel eluate fraction brought about the conversion of guanine to G5P with the simultaneous removal of PRPP.[3] In the first experiment,[4] under conditions similar to those used for A5P formation from adenine (Assay A), the disappearance of 0.12 μmole of PRPP was matched by the chromatographic isolation of 0.14 μmole of a radioactive, guanine-containing fraction. The specific activity of this fraction (1.58×10^5 c.p.m. per μmole) was comparable to that of the guanine (1.43×10^5).

TABLE V

Non-Identity of A5P and I5P Pyrophosphorylases

Four 1.0 ml. aliquots of an aluminum hydroxide gel eluate fraction were mixed with 0.0, 0.3, 0.4, and 0.5 ml. of calcium phosphate gel (17 mg. of dry weight per ml.), respectively, and then made to 1.5 ml. with water. After 5 minutes at 2°, the mixtures were centrifuged and the supernatant fluids were assayed for A5P production or residual hypoxanthine, depending on whether adenine or hypoxanthine was the substrate used. 1 unit of enzyme is defined as that amount causing the formation of 1 μmole of product or the disappearance of 1 μmole of substrate per hour.

	Gel addition, mg. of solid			
	0	5.1	6.8	8.5
	Enzyme activity, units per ml.			
Adenine as substrate	2.26	1.40	0.93	0.56
Hypoxanthine as substrate	1.20	0.30	0.07	0.03
$\frac{\text{Adenine activity}}{\text{Hypoxanthine activity}}$	1.9	4.7	13.1	18.7

In a second experiment, similar to the first, but on 3 times the scale, a comparable fraction contained 0.44 μmole of guanine as determined by

[3] The rate of this reaction was approximately 0.50 μmole per hour per ml. (of this enzyme fraction) as compared with values of 2.26 and 1.20 for adenine and hypoxanthine, respectively, as substrates.

[4] The incubation mixture (4.0 ml.) contained 0.08 ml. of Tris buffer (1 M, pH 8.0), 0.08 ml. of phosphate buffer (1 M, pH 7.4), 0.4 ml. of PRPP (0.001 M), 2.0 ml. of guanine-8-C^{14} (1.8×10^{-4} M, 1.5×10^5 c.p.m. per μmole), 0.08 ml. of $MgCl_2$ (0.1 M), and 0.32 ml. of aluminum hydroxide gel eluate fraction. After incubation for 45 minutes at 36°, the enzyme was inactivated by heating for 1 minute at 100° and the mixture was chromatographed on a Dowex 1 column, chloride form (3 cm. × 1 sq. cm.), at 2°. After 15 resin bed volumes of 0.002 N HCl (sufficient to elute the guanine), the eluent was replaced by 0.005 N HCl-0.025 M KCl. The radioactive fraction appeared after 11 resin bed volumes and was completely eluted in the next 4. In a subsequent experiment, a 60 ml. incubation mixture was chromatographed on a 3 cm. × 3.5 sq. cm. column with comparable results.

ultraviolet absorption and 0.43 μmole of pentose. In a third similar experiment, but on 15 times the scale of the first, 2.80 μmoles of the guanine-containing compound were isolated and demonstrated to be the 5′-substituted phosphate ester of guanosine by the action of purified 5-nucleotidase (11).[5] It has not been determined whether the G5P-synthesizing activity of the enzyme preparation is distinct from the A5P- and I5P-forming systems described above.

TABLE VI

Balance Study of I5P Synthesis

The experimental incubation mixture (10.0 ml.) contained 1.0 ml. of hypoxanthine (0.0014 M), 1.0 ml. of PRPP (0.001 M), 0.2 ml. of Tris buffer (1 M, pH 8.0), 0.2 ml. of potassium phosphate buffer (1 M, pH 7.4), 0.2 ml. of $MgCl_2$ (0.1 M), and 0.8 ml. of the enzyme (aluminum hydroxide gel eluate fraction). After incubation at 36° for 45 minutes, the enzyme was inactivated by heating the mixture in a boiling water bath for 1 minute. Two control incubation mixtures, one lacking only hypoxanthine and the other only PRPP, were incubated and then the missing component was added after inactivation of the enzyme. The values in bold-faced type were obtained by chromatography of 9.0 ml. of the reaction mixtures (adjusted to pH 8.5) on a Dowex 1 column, chloride form (3 cm. × 1 sq. cm.); the first eluent was 0.001 N HCl for 50 resin bed volumes (sufficient to elute hypoxanthine); the second eluent was 0.005 N HCl-0.05 M KCl, which promptly eluted I5P. The other values were obtained by direct enzymatic assay of aliquots of the incubation mixtures. The values are expressed in micromoles.

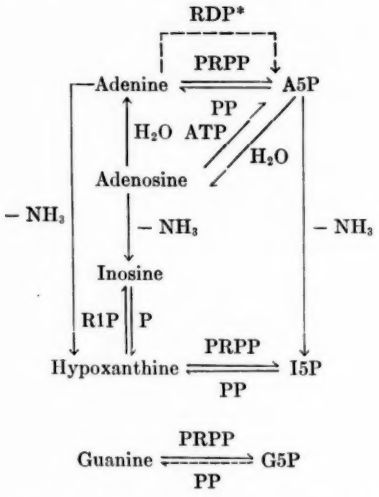
	Control	Experimental	Δ
Hypoxanthine	1.34	0.78	−0.56
"	**0.99**	**0.51**	**−0.48**
PRPP	0.78	0.38	−0.40
I5P	**0.00**	**0.48**	**+0.48**

DISCUSSION

These studies describe a pathway of purine nucleotide synthesis which can be operative in yeast and liver cells. The existence in these cells of

[5] Guanosine liberation rather than phosphate mineralization was used to evaluate 5-nucleotidase action, since the guanine-containing fraction was contaminated with PP. The incubation mixture (2.5 ml.) contained 0.02 ml. of 5-nucleotidase, 2.0 ml. of the unknown fraction (containing 0.28 μmole of C^{14}-guanine), 0.2 ml. of glycine (1 M, pH 8.5), and 0.1 ml. of $MgCl_2$ (0.1 M). After incubation for 60 minutes at 36°, the enzyme was inactivated by heating for 2 minutes at 100° and the mixture was chromatographed on a Dowex 1 column, chloride form (3 cm. × 1 sq. cm.). All of the radioactivity, representing 94 per cent of that in the incubation mixture, appeared in a single fraction between 4 and 5 resin bed volumes of eluent (0.005 N HCl, 0.025 M KCl); guanosine appeared in this fraction also when chromatographed under identical conditions. In a control run with preheated enzyme, all the radioactivity appeared in a later fraction (7 to 9 resin bed volumes).

adenine and adenosine deaminases (12), inosine phosphorylase (13), adenosine kinase (14), and enzymes which form ribotides of acyclic purine precursors (15) makes it evident that purine nucleotides can be formed by other mechanisms as well (Scheme 1).



SCHEME 1. Possible pathways of purine conversion to nucleotides. *, condensation of RDP (ribose-1,5-diphosphate) with adenine (16) is regarded as tentative (1).

An observation which is difficult to interpret and requires further study is the feeble and limited pyrophosphorolysis of A5P and I5P. The fact that the equilibrium point of orotidylic acid pyrophosphorolysis actually favors cleavage (2) makes it unlikely that the limited conversion of the purine nucleotides is the result of an unfavorable equilibrium. Perhaps the required presence of relatively high concentrations of PP or of F, to inhibit the PPase in the enzyme preparations, results in a profound inhibition of the enzyme.

SUMMARY

1. An enzyme has been partially purified from yeast which catalyzes the reaction:

Adenine + 5-phosphoribosylpyrophosphate (PRPP) ⇄

adenosine-5′-phosphate + inorganic pyrophosphate (PP)

This activity appears to be present also in liver.

2. Analogous reactions have been observed with hypoxanthine and guanine as substrates.

$$\text{Hypoxanthine} + \text{PRPP} \rightleftarrows \text{inosine-5'-phosphate} + \text{PP}$$

$$\text{Guanine} + \text{PRPP} \rightleftarrows \text{guanosine-5'-phosphate} + \text{PP}$$

The adenine and hypoxanthine enzymes have been found to be distinct; the nature of the guanine enzyme has not been determined.

3. The affinities of adenine and PRPP for the enzyme have been shown to be relatively high ($K_m < 4 \times 10^{-5}$ M).

BIBLIOGRAPHY

1. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 389 (1955).
2. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 403 (1955).
3. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2027 (1954).
4. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69,** 1382 (1947).
5. Clark, V. M., and Kalckar, H. M., *J. Chem. Soc.*, 1029 (1950).
6. Kunitz, M., *J. Gen. Physiol.*, **35,** 423 (1951–52).
7. Kalckar, H. M., *J. Biol. Chem.*, **167,** 429 (1947).
8. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 429 (1955).
9. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56,** 1117 (1923).
10. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124,** 397 (1938).
11. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **188,** 665 (1951).
12. Roush, A. H., *Arch. Biochem. and Biophys.*, **50,** 510 (1954).
13. Kalckar, H. M., *J. Biol. Chem.*, **167,** 477 (1947). Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **198,** 683 (1952).
14. Caputto, R., *J. Biol. Chem.*, **189,** 801 (1951). Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193,** 481 (1951).
15. Buchanan, J. M., and Wilson, D. W., *Federation Proc.*, **12,** 646 (1953). Greenberg, G. R., *Federation Proc.*, **12,** 651 (1953). Goldthwait, D. A., and Peabody, R. A., *Federation Proc.*, **13,** 218 (1954).
16. Saffran, M., and Scarano, E., *Nature*, **172,** 949 (1953).

ENZYMATIC SYNTHESIS OF NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES*

By IRVING LIEBERMAN, ARTHUR KORNBERG, AND ERNEST S. SIMMS

(From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, November 22, 1954)

It has been recognized from recent studies that nucleoside triphosphates, other than ATP,[1] function in biologic systems as phosphorylating agents (1) and in coenzyme synthesis (2) in a manner analogous to that of ATP. The discovery of the natural occurrence of a variety of purine and pyrimidine nucleoside di- and triphosphates (3–5) and the likelihood that new biologic functions for these nucleotides will be found increased our interest in the mechanism of their biosynthesis. Several pathways are known for the formation of UTP, including its origin from UDP, by the action of pyruvate phosphokinase (6) or of nucleoside diphosphate kinase (7), but no mechanism has been elucidated for the synthesis of UDP or UTP from U5P. In a preliminary communication (8), we have reported that extracts of yeast and liver which convert orotic acid to U5P also bring about the synthesis of UDP and UTP. A subsequent report (9) described the action of a partially purified enzyme preparation from yeast which effects a transphosphorylation between adenosine, uridine, and guanosine nucleotides (equations (1) to (5)) and differs from muscle myokinase (10, 11) which acts on adenosine nucleotides only.

(1) $$U5P + ATP \rightleftharpoons UDP + ADP$$

(2) $$A5P + UTP \rightleftharpoons ADP + UDP$$

(3) $$U5P + UTP \rightleftharpoons 2UDP$$

(4) $$G5P + ATP \rightleftharpoons GDP + ADP$$

(5) $$2GDP \rightleftharpoons GTP + G5P$$

* This investigation was supported by research grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

[1] The abbreviations used are adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine-5′-phosphate, A5P; guanosine triphosphate, GTP; guanosine diphosphate, GDP; guanosine-5′-phosphate, G5P; inosine triphosphate, ITP; inosine diphosphate, IDP; uridine triphosphate, UTP; uridine diphosphate, UDP; uridine-5′-phosphate, U5P; diphosphopyridine nucleotide, DPN; reduced DPN, DPNH; triphosphopyridine nucleotide, TPN; tris(hydroxymethyl)aminomethane, Tris.

XUM

These reactions can be summarized by a generalized equation for the action of a nucleoside monophosphate kinase (equation (6)).

(6) Nucleoside-P + *nucleoside*-P-P-P $\rightleftharpoons$ nucleoside-P-P + *nucleoside*-P-P

This report provides the details of the partial purification and properties of the enzyme preparation from yeast which catalyzes the formation of the nucleoside di- and triphosphates. Preceding reports in this series (12–14) have described a pathway of synthesis of pyrimidine and purine nucleotides from their constituent bases.

Materials

2-C^{14}-U5P was synthesized from 2-C^{14}-sodium orotate as previously described (13) and concentrated by adsorption on and elution from Norit. 2-C^{14}-A5P and 2-C^{14}-G5P were prepared enzymatically from the corresponding 2-C^{14}-labeled purine bases (14) and purified by anion exchange chromatography. U5P, UTP, and ATP were purified by anion exchange chromatography. ATP^{32} was prepared as previously described (12). Deoxynucleotides were products of the California Foundation for Biochemical Research. Nicotinamide ribose-5′-phosphate was prepared according to Kornberg and Pricer (15).

IDP was prepared from ITP in the presence of glucose and a catalytic amount of ADP by the combined action of nucleoside diphosphokinase (7) and hexokinase (16). The reaction was measured spectrophotometrically by the reduction of TPN in the presence of glucose-6-phosphate dehydrogenase (17). After complete conversion of the ITP (0.16 μmole per ml.), the reaction mixture was heated in a boiling water bath for 3 minutes; the supernatant solution, after centrifugation, was used as IDP.

TPN was obtained by anion exchange chromatography of a crude liver fraction (18). DPNH was prepared by Ohlmeyer's method (19) and phosphopyruvic acid by Ohlmeyer's modification of Kiessling's procedure (20).

Pyruvate phosphokinase was purified from rabbit muscle by a modification of the procedure described by Kubowitz and Ott (21). Lactic dehydrogenase was prepared by ammonium sulfate fractionation of rabbit muscle extract (22), hexokinase according to Berger *et al.* (16), and glucose-6-phosphate dehydrogenase by the method of Kornberg (17). Adenylic acid deaminase was prepared from rabbit muscle by the procedure used for Preparation A by Kalckar (23).

Determinations

Enzyme Assay—This was based on the conversion of C^{14}-labeled U5P to UDP and UTP. The reaction mixture (1.0 ml.) contained 0.1 ml. of glycylglycine buffer (0.5 M, pH 7.5), 0.05 ml. of $MgCl_2$ (0.1 M), 0.1 ml. of

ATP (0.04 M), 0.01 ml. of potassium phosphopyruvate (0.05 M), 4 units of pyruvate phosphokinase, 0.05 ml. of 2-C^{14}-U5P (6.8×10^{-4} M, 240,000 c.p.m. per μmole), and 0.6 to 2.6 units of enzyme. After 15 minutes at 36°, 1 ml. of water was added and the diluted mixture was heated in a boiling water bath for 3 minutes. The supernatant fluid obtained by centrifugation was adsorbed on a column of Dowex 1, chloride form (200 to 400 mesh, 2 per cent cross-linked; height 1.5 cm., diameter 1 cm.), contained in a columnar glass funnel with a coarse sintered glass disk. After the fluid was drawn through the column with gentle suction, the resin was washed first with 2.5 ml. of water and then with 25 ml. of HCl (0.05 N) (at a rate of about 1.5 ml. per minute) to elute the U5P. Up to six such columns were readily handled at the same time by setting up a glass manifold to connect the HCl reservoir to the columns. The uridine polyphosphates were then eluted with 4 N HCl (2.5 ml.) with the aid of suction. An aliquot (0.2 ml.) of this eluate was plated on a circular glass cover-slip (diameter 2.2 cm.), dried, and counted. A unit of enzyme was defined as the amount producing 1000 c.p.m. in the strong HCl eluate under the conditions of the assay. Specific activity was defined as units of activity per mg. of protein.

Under the assay conditions, the radioactivity of the strong HCl eluate was proportional to the amount of enzyme. Thus, assays of 0.010, 0.015, and 0.020 ml. of enzyme solution all yielded values of 80 units per ml. No counts were detected in the absence of enzyme.

Chromatographic analysis of the amounts of UDP and UTP in the strong HCl eluate under standard assay conditions with a purified enzyme fraction indicated a ratio of 1:4. Under similar conditions but with one-twentieth as much ATP (0.2 μmole per ml.), roughly equal amounts of UDP and UTP were produced.

Adenylate kinase was measured with the coupled pyruvate phosphokinase-lactic dehydrogenase system as an indicator (22). The reaction mixture (1.0 ml.) contained 0.1 ml. of glycylglycine buffer (0.5 M, pH 7.5), 0.05 ml. of $MgCl_2$ (0.1 M), 0.01 ml. of potassium phosphopyruvate (0.05 M), 0.05 ml. of DPNH (0.001 M), 0.01 ml. of lactic dehydrogenase (diluted 1:5000), 5 units of pyruvate phosphokinase, 0.1 ml. of ATP (3.5×10^{-4} M), 0.1 or 0.2 ml. of enzyme solution, and 0.02 ml. of A5P (0.025 M). The formation of ADP was calculated as a function of pyruvate appearance and measured by the decrease in optical density at 340 mμ resulting from the oxidation of DPNH (equations (7) to (9)).

(7) $$\text{ATP} + \text{A5P} \rightleftharpoons 2\text{ADP}$$

(8) $$\text{Phosphopyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$$

(9) $$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}$$

Nucleoside diphosphokinase was assayed with ATP and IDP in the

presence of yeast adenylate kinase and adenylic acid deaminase as described by Berg and Joklik (7).

ADP and UDP were estimated spectrophotometrically with the phosphokinase-lactic dehydrogenase system (22).

Pentose, phosphate, and protein were determined by methods previously cited (12).

C^{14}-containing samples were plated as thin layers on aluminum disks or, in the presence of strong acid, on glass cover-slips. Radioactivity was measured in a gas flow counter. When a large amount of salt was present, a self-absorption correction factor was applied (1.25 for 0.05 M KCl; 1.35 for 0.1 M KCl). P^{32} was measured in solution (1.0 ml.) in a dish under a Geiger-Müller tube.

Results

Purification of Enzyme—The enzyme catalyzing the synthesis of UDP and UTP from U5P and ATP was purified about 30-fold from extracts of yeast, as shown in Table I. The operations were carried out at 0° except as indicated.

Extracts were prepared from dried brewers' yeast by incubation in 0.1 M $KHCO_3$ for 5 hours at 30° as previously described (13).

To 36 ml. of yeast autolysate, 72 ml. of sodium acetate buffer (0.2 M, pH 4.5) were added rapidly, with stirring, and the acidified extract was cooled to −2° in an alcohol-ice bath. 45 ml. of ethanol (−14°) were then added over a 7 minute interval while the temperature was allowed to fall to −4°. The mixture was cooled to −8° and a further addition of ethanol was made (29 ml.) over a 4 minute interval at −8°. Insoluble material was removed by centrifugation for 3 minutes at 10,000 × *g* in a Servall centrifuge kept in a room at −14°. To the supernatant fluid, 90 ml. of ethanol were added over a 4 minute interval with the temperature maintained at −10°. The precipitate was collected by centrifugation (3 minutes at 10,000 × *g*), dissolved in glycylglycine buffer (0.025 M, pH 7.0), adjusted to pH 7.0 with 1 N KOH, and brought to a final volume of 36 ml. (ethanol fraction).

To the ethanol fraction (36 ml.) were added 1.44 ml. of Tris buffer (0.1 M, pH 8.0) and sufficient calcium phosphate gel (24) (260 mg. of solids) to adsorb 40 to 50 per cent of the activity. After 5 minutes, the supernatant fluid was collected by centrifugation (calcium phosphate fraction).

To the calcium phosphate fraction (50 ml.) was added sufficient aged alumina gel Cγ (25) (22.5 mg. of solids) to adsorb 90 to 95 per cent of the activity. The gel was washed with 10 ml. of potassium phosphate buffer (0.01 M, pH 7.2) and the enzyme was then eluted with 10 ml. of sodium pyrophosphate buffer (0.03 M, pH 8.5) (aluminum hydroxide gel fraction).

Survey of Nucleoside Monophosphate Kinase Activities of Enzyme Preparation—ATP was tested as phosphate donor with a number of nucleoside monophosphates in the spectrophotometric assay system described for adenylate kinase (see above). A5P, U5P, G5P, and deoxyadenosine-5′-phosphate were active as acceptors; no activity was observed with inosine-5′-phosphate, deoxycytidine-5′-phosphate, deoxyguanosine-5′-phosphate, nicotinamide ribose-5′-phosphate, or adenosine-3′-phosphate. With UTP as donor, A5P and U5P proved to be acceptors. While UTP can be tested as a phosphorylating agent in this assay procedure (*i.e.*, UDP participates in the pyruvate phosphokinase reaction but at a much slower rate than ADP (6)), the result is far less sensitive than with ATP and negative findings are less meaningful.

Stoichiometry in Transphosphorylation between UTP and U5P—When 2-C^{14}-U5P and UTP were incubated with the purified enzyme, UDP was

TABLE I

Purification of Enzyme

Enzyme fraction	Total units	Over-all recovery	Specific activity
		per cent	*units per mg. protein*
Autolysate	2860	100	5.3
Ethanol fraction	1410	49.3	25.1
Calcium phosphate fraction	707	24.7	53.2
Aluminum hydroxide gel fraction	591	20.6	174.0

formed and approximately equal amounts of U5P and UTP disappeared (Table II). The UDP was isolated chromatographically[2] and identified by its absorption spectrum (maximum at 262 mμ, λ280/λ260 = 0.37 in 0.01 N HCl) and by the molar ratios of uracil to acid-labile phosphate to total phosphate to phosphate acceptor (in the phosphopyruvate-pyruvate phosphokinase system) of 1.00:0.97:1.94:0.94. In the absence of either U5P or UTP, no UDP was formed.

The high specific radioactivity of the UTP and the low value for UDP (Table II) cannot be explained by equation (3), but they could be the result of the action of nucleoside diphosphokinase, which was observed to be

[2] The preparation and isolation of UDP in a larger amount were carried out with a 10 ml. reaction mixture, in which the amounts of U5P, UTP, and enzyme were increased 10-fold and the other components 5-fold. After incubation for 2 hours at 36°, the mixture was heated in a boiling water bath for 3 minutes and then chromatographed on a column of Dowex 1, chloride form (200 to 400 mesh, 2 per cent cross-linked, 5 cm. high, 1 cm. diameter) at 3°. 7.0 μmoles of UDP appeared between 37 and 60 resin bed volumes of 0.01 N HCl-0.05 M KCl. The eluate was concentrated by adsorption on and elution from Norit.

present in this enzyme preparation. When the experiment was repeated[3] with an enzyme preparation that had been freed of nucleoside diphosphokinase by heating at low pH, the specific radioactivity of the UTP was reduced to less than 5 per cent of that of the UDP.

Equilibrium Constant for Uridylate Kinase Reaction—Starting with UTP and U5P on the one hand, or with UDP on the other, steady state concentrations were reached which appeared to represent an equilibrium point (Table III). Constants calculated in the direction of UDP formation in the experiment starting with UTP and U5P or with UDP were 0.92 and 0.95, respectively.

TABLE II

Stoichiometry of UDP Synthesis from U5P and UTP

The reaction mixture (2.0 ml.) contained 0.2 ml. of glycylglycine buffer (0.5 M, pH 7.5), 0.1 ml. of $MgCl_2$ (0.1 M), 0.2 ml. of 2-C^{14}-U5P (0.01 M, 17,000 c.p.m. per μmole), 0.45 ml. of UTP (0.004 M), and 44.3 units of enzyme (specific activity 174). After incubation at 36° for 45 minutes, the reaction mixture was heated in a boiling water bath for 3 minutes and chromatographed on a column of Dowex 1, chloride form (200 to 400 mesh, 2 per cent cross-linked; height 2 cm., diameter 1 cm.). U5P was eluted with a solution of 0.01 N HCl-0.05 M KCl, UDP with 0.01 N HCl-0.1 M KCl, and UTP with 0.1 N HCl-0.1 M KCl. The fractions were analyzed spectrophotometrically and by radioactivity measurement.

	0 min.		45 min.		Δ	
	μmoles	*c.p.m. per μmole*	*μmoles*	*c.p.m. per μmole*	*μmole*	*total c.p.m.*
U5P	2.20	17,000	1.81	15,430	−0.39	−9270
UTP	1.64	0	1.27	4,110	−0.37	+5220
UDP	0.00	0	0.82	6,500	+0.82	+5330

Transphosphorylation between UTP and A5P—A balance study of the transphosphorylation between adenosine and uridine nucleotides was carried out with 2-C^{14}-A5P and UTP and an enzyme preparation which had been heated at acid pH (Table IV). Chromatographic analysis of the reaction mixture showed that the disappearance of 0.59 μmole of A5P was matched by the appearance of 0.61 μmole (combined) of ADP and ATP and the disappearance of 0.51 μmole of UTP by the appearance of 0.66 μmole (combined) of U5P and UDP. No reaction occurred when either A5P or UTP was omitted from the reaction mixture.

[3] The reaction mixture was the same as that shown in Table II, except for the specific activity of the U5P (58,000 c.p.m. per μmole) and the use of 30.1 units of enzyme which had been heated at pH 2.1 in a boiling water bath. 0.13 μmole each of U5P and UTP disappeared, and 0.27 μmole of UDP was formed. The specific activities of the nucleotides were found to be 55,300 for U5P, 1115 for UTP, and 24,450 c.p.m. per μmole for UDP.

Synthesis of Guanosine Triphosphate—On the basis of spectrophotometric evidence for the phosphorylation of G5P (see above) an attempt was made to isolate and identify the products.

The reaction mixture (50 ml.) contained 4.5 ml. of glycylglycine buffer (0.5 M, pH 7.5), 2.25 ml. of $MgCl_2$ (0.1 M), 15.0 ml. of ATP (2.7×10^{-4} M),

TABLE III

Equilibrium Concentrations of Uridine Nucleotides

For UDP synthesis, each reaction mixture contained (in 1.5 ml.) 0.2 ml. of glycylglycine buffer (0.5 M, pH 7.5), 0.1 ml. of $MgCl_2$ (0.1 M), 0.05 ml. of U5P (0.01 M), 0.11 ml. of UTP (0.004 M), and 40 units of enzyme (specific activity 235). After incubation of three such mixtures at 36° for 50 minutes, one was heated at 100° for 3 minutes and 25 units of enzyme were added to each of the other two. At 100 minutes, one of these mixtures was heated as above, and 25 units of enzyme were added to the remaining mixture. The latter was heated at 150 minutes. *For UDP dismutation*, each reaction mixture contained (in 1.5 ml.) 0.4 ml. of UDP (1.9×10^{-3} M), buffer, $MgCl_2$, and enzyme as above. After incubation of two such mixtures at 36° for 50 minutes, an additional 25 units of enzyme were added to each. At 120 minutes one reaction mixture was heated at 100° and 25 units of enzyme were added to the remaining mixture. The reaction was stopped in this reaction mixture at 180 minutes by heating. The entire reaction mixtures were chromatographed on columns of Dowex 1, chloride form (2 per cent cross-linked; height 2 cm., diameter 1 cm.), and eluted as described in Table II. The uridine nucleotides were characterized by their chromatographic behavior and estimated spectrophotometrically at 260 mμ.

Products	UDP synthesis, μmole				UDP dismutation, μmole		
	0 min.	50 min.	100 min.	150 min.	0 min.	120 min.	180 min.
U5P	0.50*	0.41	0.36	0.34		0.24	0.27
UDP		0.17	0.26	0.29	0.76*	0.26	0.25
UTP	0.44*	0.31	0.28	0.27		0.21	0.24
$K_{eq.}$†				0.92			0.95

* Calculated values.

† Calculated in the direction of UDP synthesis.

15.0 ml. of 2-C^{14}-G5P (8×10^{-5} M, 15,800 c.p.m. per μmole), and 300 units of enzyme (ethanol fraction, heated for 3 minutes at pH 1.7 in a boiling water bath). In order to maintain a high level of ATP, 0.25 ml. of phosphopyruvate (0.05 M) and 5 units of pyruvate phosphokinase were also added. After incubation at 36° for 1 hour, the reaction mixture was heated in a boiling water bath for 3 minutes and then rapidly cooled. The entire reaction mixture was then chromatographed at 3° on a column of Dowex 1, chloride form (2 per cent cross-linked; height 3 cm., diameter 1 cm.). To elute any unchanged G5P, the column was first treated with 34

XUM

resin bed volumes of a solution of 0.01 N HCl-0.05 M KCl. Only negligible amounts of radioactivity were eluted and the eluent was changed to 0.01 N HCl-0.1 M KCl.[4] The product, presumed to be GTP, was detected by radioactivity and spectrophotometric measurements and appeared as a

TABLE IV

Transphosphorylation between Uridine and Adenosine Nucleotides

The reaction mixture (2.05 ml.) contained 0.2 ml. of glycylglycine buffer (0.5 M, pH 7.5), 0.1 ml. of $MgCl_2$ (0.1 M), 0.5 ml. of 2-C^{14}-A5P (0.002 M, 82,000 c.p.m. per μmole), 0.25 ml. of UTP (0.004 M), and 16 units of heated enzyme (ethanol fraction heated for 3 minutes at pH 1.5 in a boiling water bath; specific activity 28.3). After incubation at 36° for 1 hour, the reaction mixture was heated in a boiling water bath for 3 minutes. The entire reaction mixture was chromatographed on a column of Dowex 1, chloride form (2 per cent cross-linked; height 2 cm., diameter 1 cm.), at 3°. A5P was eluted with 0.01 N HCl. U5P and ADP were eluted together with 0.01 N HCl-0.05 M KCl, and UDP (separated from the other nucleotides except in a few ATP-containing fractions) was eluted with the same solvent. ATP was eluted with 0.01 N HCl-0.1 M KCl and UTP with a solution of 0.1 N HCl-0.1 M KCl. A5P was estimated spectrophotometrically at 260 mμ and by radioactivity measurements and ADP by radioactive measurements and with pyruvate phosphokinase (0.29 μmole found). ATP was measured by radioactivity, by spectrophotometric determinations, and by enzymatic assay (17). U5P was estimated spectrophotometrically at 260 mμ, with a correction for the ADP present. UDP was likewise measured spectrophotometrically (260 mμ), with a correction for the ATP present, and, in addition, the two peak fractions were estimated with pyruvate phosphokinase (6). Spectrophotometric measurements showed the presence of 0.09 and 0.11 μmole and the enzymatic assay 0.08 and 0.10 μmole of UDP in the two fractions, respectively. UTP was estimated spectrophotometrically at 260 mμ.

	0 min.	60 min.	Δ	
	μmole	*μmole*	*μmole*	*total c.p.m.*
A5P	0.89	0.30	−0.59	−45,260
UTP	0.83	0.32	−0.51	
ADP	0.00	0.33	+0.33	+26,800
ATP	0.00	0.28	+0.28	+23,100
U5P	0.00	0.31	+0.31	
UDP	0.00	0.35	+0.35	

discrete, symmetrical peak between 1.9 and 14.6 resin bed volumes of the stronger eluent, with a peak at 7.4 volumes. It represented at least 70 per cent of the counts applied to the column. GTP was identified by its absorption spectrum ($\lambda 250/\lambda 260 = 1.0$, $\lambda 280/\lambda 260 = 0.66$, peak at 255 mμ, in 0.01 N HCl; $\lambda 250/\lambda 260 = 1.12$, $\lambda 280/\lambda 260 = 0.55$, at pH 7) and by the

[4] ATP was eluted in part with the weaker eluent (first appearing at 11.9 resin bed volumes of eluent) and was completely removed in the early fractions of the stronger eluent (0 to 5.6 resin bed volumes of this eluent).

molar ratios of guanine to pentose to acid-labile phosphate to total phosphate of 1.00:0.96:2.02:3.02. The specific activity of the GTP was 15,700 c.p.m. per μmole.[5]

In a second experiment carried out with G5P (0.04 μmole), ATP^{32} (0.14 μmole, 7×10^6 c.p.m. per μmole), and a similar enzyme preparation, but without phosphopyruvate and pyruvate phosphokinase, chromatographic analysis of the reaction mixture revealed two peaks not present when G5P was omitted from the reaction mixture. The first peak (39,000 c.p.m.) appeared between 13 and 20 resin bed volumes of eluent (0.01 N HCl-0.05 M KCl) and was presumed to be GDP. The second peak (97,200 c.p.m.), considered to be GTP, was eluted with a stronger eluent (0.01 N HCl-0.1 M KCl) and appeared immediately after ATP with a peak at 12 resin bed volumes of this eluent.

TABLE V

Differentiation of Uridylate and Adenylate Kinases by Heat Lability

The heating was for 3 minutes in a boiling water bath.

	Uridylate kinase remaining	Adenylate kinase remaining
	per cent	*per cent*
pH 7	<1	14.6
" 7 + Mg^{++} (0.01 M)	<2	36.9

Differentiation of Enzyme from Adenylate Kinase—The activity responsible for the nucleotide transphosphorylation reactions described above was relatively stable to heating at low pH. Thus, after incubation of the ethanol fraction for 3 minutes in a boiling water bath at pH 1.3, 2.7, or 3.7, 53.2, 50.1, and 39.4 per cent, respectively, of the original mixed myokinase activities were recovered. While the adenylate kinase activity of yeast is also relatively stable to heating at acid pH (22), the two enzymes were readily distinguished by their heat stabilities at neutral pH (Table V).

Influence of pH, Mg^{++}, and KCl on Enzyme—Maximal rates of UDP + UTP formation were observed (under standard assay conditions, except that Tris-maleate buffer replaced glycylglycine) around pH 7.9. At pH values of 5.0, 5.6, 6.7, and 9.5, the rates were 24, 37, 63, and 87 per cent, respectively, of the maximal value. In the absence of added Mg^{++}, the rate was 18 per cent of that observed with 0.005 M Mg^{++}. With KCl at a

[5] To avoid an absorption correction in radioactivity measurements, an aliquot of one of the fractions (1.5 ml.) was desalted by passing it through a column of Dowex 50 (H^+) resin bed volume of 1.0 ml. An aliquot of the desalted sample was dried on a glass cover-slip and counted.

concentration of 0.5 M, there was only a 30 per cent inhibition of the rate, and at a level of 0.2 M there was no effect at all.

DISCUSSION

The results of these studies indicate that UTP can be formed from U5P and ATP and that ATP can be formed from A5P and UTP. It seems clear that the mechanism is essentially similar to that of the adenylate kinase ("myokinase") reaction (10, 11): there is a reversible transphosphorylation of the terminal phosphate from a nucleoside triphosphate to a nucleoside monophosphate to form two nucleoside diphosphates. It has been shown that the adenylate kinase of muscle is strictly specific for adenosine nucleotides and it is evident from our studies that there is an adenylate kinase in yeast with a similar specificity. It is now apparent that another enzyme (or possibly several enzymes) is present in yeast[6] which catalyzes a transphosphorylation between uridine and adenosine nucleotides, guanosine and adenosine nucleotides, and apparently between uridine nucleotides. With regard to the latter reaction (U5P + UTP $\rightleftharpoons$ 2UDP), the possibility exists that an adenosine nucleotide, as a trace contaminant in one of the reaction components, could catalytically effect a primary transphosphorylation with a uridine nucleotide. Thus far in our studies with yeast[7] there is no evidence for the participation of an adenosine nucleotide in this reaction, but a rigorous test of this possibility must await further purification of the enzyme preparation. Such purification may also be helpful in determining whether a single enzyme with a wide range of specificity among the nucleotides, or a family of enzymes with restricted specificities, is responsible for the observed transphosphorylations.

SUMMARY

1. A partially purified enzyme preparation from yeast has been observed to catalyze transphosphorylations between adenosine, uridine, and guanosine nucleotides. The various nucleoside di- and triphosphates appeared

[6] Similar reactions appear to have been observed by others with animal tissues (26) and in yeast (27).

[7] Strominger, Heppel, and Maxwell observed no reaction with purified UDP preparations in their calf liver enzyme system. We found a UDP sample, which they kindly furnished us, to be active with our yeast enzyme. When 1.3 μmoles of this UDP preparation were incubated with 47.7 units of the partially purified yeast enzyme (aluminum hydroxide gel fraction, see Table I) for 105 minutes, the disappearance of 0.54 μmole of UDP was accompanied by the appearance of 0.32 and 0.28 μmole of U5P and UTP, respectively. Under the same conditions, incubation of the U5P (1.32 μmoles) and of UTP (0.78 μmole) preparations used in previous experiments yielded 0.50 μmole of UDP.

to be formed by a mechanism summarized by the general equations

(*a*) Nucleoside-P + *nucleoside*-P-P-P $\rightleftharpoons$ nucleoside-P-P + *nucleoside*-P-P

(*b*) 2 nucleoside-P-P $\rightleftharpoons$ nucleoside-P + nucleoside-P-P-P

(*c*) 2 *nucleoside*-P-P $\rightleftharpoons$ nucleoside-P + *nucleoside*-P-P-P

These reactions provide a mechanism, hitherto unavailable, for the biosynthesis of UTP, GTP, and probably other nucleoside triphosphates from their respective monophosphates.

2. The enzyme (or enzymes) responsible for these reactions was distinguished from yeast adenylate kinase ("myokinase") by its lability to heating at neutral pH; like adenylate kinase, it is relatively stable to heating at acid pH.

3. Equilibrium constants and balance data for some of these reactions were determined.

BIBLIOGRAPHY

1. Utter, M. F., Kurahashi, K., and Rose, I. A., *J. Biol. Chem.*, **207,** 803 (1954). Ling, K.-H., and Lardy, H. A., *J. Am. Chem. Soc.*, **76,** 2842 (1954).
2. Munch-Petersen, A., Kalckar, H. M., Cutolo, E., and Smith, E. E. B., *Nature*, **172,** 1036 (1953).
3. Bergkvist, R., and Deutsch, A., *Acta chem. Scand.*, **7,** 1307 (1953).
4. Potter, V. R., Hurlbert, R. B., and White, D. M., *Cancer Res.*, **14,** 66 (1954).
5. Schmitz, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **209,** 41 (1954).
6. Kornberg, A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 392 (1951).
7. Berg, P., and Joklik, W. K., *Nature*, **172,** 1008 (1953). Krebs, H. A., and Hems, R., *Biochim. et biophys. acta*, **12,** 172 (1953).
8. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2027 (1954).
9. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 3608 (1954).
10. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148,** 117 (1943).
11. Kalckar, H. M., *J. Biol. Chem.*, **148,** 127 (1943).
12. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 389 (1955).
13. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Biol. Chem.*, **215,** 403 (1955).
14. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215,** 417 (1955).
15. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182,** 763 (1950).
16. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29,** 379 (1946).
17. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
18. Kornberg, A., and Horecker, B. L., *Biochem. Preparations*, **3,** 20 (1953).
19. Ohlmeyer, P., *Biochem. Z.*, **297,** 66 (1938).
20. Ohlmeyer, P., *J. Biol. Chem.*, **190,** 21 (1951).
21. Kubowitz, F., and Ott, P., *Biochem. Z.*, **317,** 193 (1944).
22. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193,** 481 (1951).
23. Kalckar, H. M., *J. Biol. Chem.*, **167,** 461 (1947).

XUM

24. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
25. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, **56**, 1117 (1923).
26. Strominger, J. L., Heppel, L. A., and Maxwell, E. S., *Arch. Biochem. and Biophys.*, **52**, 488 (1954). Herbert, E., Potter, V. R., and Takagi, Y., *J. Biol. Chem.*, **213**, 923 (1955). Gibson, D. M., Ayengar, P., and Sanadi, D. R., Abstracts, American Chemical Society, 126th meeting, New York, 41C, Sept. (1954).
27. Munch-Petersen, A., *Acta chem. Scand.*, **8**, 1102 (1954).

HYDROLYSIS AND TRANSFER REACTIONS CATALYZED BY ω-AMIDASE PREPARATIONS*

By ALTON MEISTER, LEON LEVINTOW, ROBERT E. GREENFIELD, and PATRICIA A. ABENDSCHEIN

(From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, December 22, 1954)

Recent studies have shown that deamidation of glutamine and asparagine may take place by at least several types of enzymatic reactions. These include (*a*) hydrolysis of glutamine or asparagine to the respective α-aminodicarboxylic acids and ammonia (1, 2), (*b*) hydrolysis as in (*a*) but dependent upon the presence of phosphate or certain other anions for activity (3–5), (*c*) deamidation of glutamine and asparagine by mechanisms involving participation of an α-keto acid (6–9), (*d*) deamidation of glutamine to glutamate associated with the synthesis of adenosine triphosphate from adenosine diphosphate and phosphate (*i.e.*, reversal of glutamine synthesis) (10), and (*e*) deamidation of glutamine and asparagine associated with ω replacement reactions (11–16). Studies in this laboratory have shown that the deamidation reactions described under (*c*) are associated with transamination leading to formation of the α-amino acid analogous to the α-keto acid (8, 9). The formation of ammonia in the α-keto acid-dependent deamidation reactions may be ascribed to enzymatic hydrolysis of the α-keto acid ω-amides, α-ketoglutaramic and α-ketosuccinamic acids, which appear to be intermediates in these reactions. Extracts of a number of tissues catalyze the hydrolysis of the α-keto acid ω-amides, and a purified enzyme preparation capable of catalyzing these reactions has been obtained from rat liver (17, 18).

The present communication describes studies on certain properties of this liver enzyme as well as those of preparations of a bacterial glutaminase and guinea pig serum asparaginase. The liver enzyme catalyzes the hydrolysis of α-ketosuccinamic, α-ketoglutaramic, succinamic, and glutaramic acids, but does not deamidate glutamine or asparagine. It also catalyzes the formation of succinylmonohydroxamic and glutarylmonohydroxamic acids from the corresponding amides and hydroxylamine much more rapidly than the hydrolysis of these amides. On the other hand, the glutaminase and asparaginase preparations studied here catalyze hydroxamic acid formation from glutamine and asparagine, respectively, at rates relatively

* Presented in part before the Division of Biological Chemistry at the 127th meeting of the American Chemical Society at Cincinnati, April 1, 1955.

low compared to those of hydrolysis. It is of interest that, in the presence of hydroxylamine, all of these preparations also catalyze monohydroxamic acid synthesis from the dicarboxylic acids corresponding to the susceptible amides. The findings are discussed in terms of the concept that the transfer and hydrolysis reactions are catalyzed by the same enzyme.

EXPERIMENTAL

Substrates—The following compounds were prepared as described: barium α-ketoglutaramate (17), sodium α-ketosuccinamate (17), sodium α-ketoadipamate (18), barium α-keto-*dl*-γ-methylglutaramate (18), D- and L-glutamine (10), D- and L-homoglutamine (18), α-methyl-DL-glutamine (18), *dl*-γ-methyl-L-glutamine (18), adipamide (19), adipamic acid (20), glutaramic acid (20), glutaramide (19), oxamic acid (21), L-γ-glutamylhydroxamic acid (10), succinamic acid,[1] sodium malonamate,[1] potassium succinylhydroxamate (22).

Acetamide, propionamide, valeramide, isovaleramide, caproamide, and malonamide were obtained from the Eastman Kodak Company. Butyramide, isobutyramide, oxamide, and succinamide were Kahlbaum products. D-Asparagine monohydrate ($[\alpha]_D^{26}$ −29.2°)[2] and L-asparagine monohydrate ($[\alpha]_D^{26}$ +29.2°)[2] were obtained from the Nutritional Biochemicals Corporation. Several of the commercial preparations required recrystallization; all of the compounds gave theoretical values for nitrogen.[3]

The authors are indebted to Dr. Jesse P. Greenstein, Dr. Sanford M. Birnbaum, and Dr. Milton Winitz of this Laboratory for the amino acid α-amides used in these studies, to Dr. Karl Pfister of Merck and Company for the α-methyl-DL-asparagine, and to Dr. Simon Black of the National Institute of Arthritis and Metabolic Diseases for a sample of L-β-aspartylhydroxamic acid.

dl-β-Methyl-DL-glutamic Acid—This compound was prepared from diethylacetamidomalonate and ethyl crotonate by a procedure analogous to those of Snyder *et al.* (23) and Done and Fowden (24) for glutamic and γ-methylglutamic acids, respectively. 3.6 gm. of sodium were added to 600 ml. of absolute ethanol, and, after the reaction was complete, 326 gm. of diethylacetamidomalonate were added and the mixture was boiled under a reflux. 208 gm. of ethyl crotonate were added dropwise over a 5 hour period, and boiling was continued for an additional 12 hours. Evaporation of the solvent yielded a white crystalline compound, which was boiled

[1] Otani, T. T., and Meister, A., Abstracts, Division of Biological Chemistry, American Chemical Society, 127th meeting, Cincinnati, April 1 (1955).

[2] 9 per cent in 3 N hydrochloric acid.

[3] The microanalyses were carried out by Dr. William C. Alford and Mr. Robert J. Koegel.

under a reflux with 10 times its weight of 6 N hydrochloric acid for 18 hours. The product was isolated and crystallized as described for γ-methylglutamic acid (18). Calculated for $C_6H_{11}O_4N$: C 44.7, H 6.9, N 8.7; found, C 44.3, H 6.8, N 8.8.

*dl-β-Methyl-*DL*-glutamine*—Carbobenzoxy-*dl*-β-methyl-DL-glutamic acid diamide was prepared and found to be resistant to deamidation by papain; the ω-amide was therefore prepared via the carbobenzoxy ω ester as described previously for α-methyl-DL-glutamine (18). Calculated for *dl*-β-methyl-DL-glutamic acid γ-ethyl ester, $C_8H_{15}O_4N$: C 50.7, H 7.9, N 7.4; found, C 50.4, H 8.2, N 7.6. Amidation of carbobenzoxy-*dl*-β-methyl-DL-glutamic acid γ-ethyl ester proceeded more slowly than did amidation of the corresponding α-methylglutamic and α-aminoadipic acid derivatives; amidation of the β-methyl compound was complete after 4 days at room temperature. Calculated for *dl*-β-methyl-DL-glutamine, $C_6H_{12}O_3N_2$: C 45.0, H 7.6, N 17.5; found, C 44.9, H 7.7, N 17.3.

dl-α-Aminomalonamic Acid—80 gm. of bromine were added dropwise over a 3 hour period to 66 gm. of malonic acid-monoethyl ester dissolved in 250 ml. of dry ether. The mixture was kept at about 25° by occasional use of an ice bath. After all the bromine was added, the mixture was stirred for an additional 30 minutes. The ether and hydrogen bromide were removed *in vacuo*, and the crude sirupy bromo compound was cooled to −10° and added to 1 liter of cold 28 per cent aqueous ammonia. The mixture was allowed to stand for 1 day in a stoppered bottle at room temperature, following which the volume was reduced to about 200 ml. by evaporation *in vacuo*. 1 liter of a 20 per cent solution of lead acetate was added, and, after standing for several hours, the precipitated lead salt was filtered and washed with cold water until the wash water was free of halogen and ammonia. The lead salt was decomposed with hydrogen sulfide, and about 30 gm. of crude α-aminomalonamic acid were obtained by evaporation of the solution to dryness *in vacuo*. Paper chromatography revealed that the product was contaminated with glycine, glycine amide, and aminomalonic acid. Attempts to recrystallize the product from warm (50°) water resulted in conversion of the desired amide to glycine and glycine amide, and some breakdown was observed, even at room temperature. Purification was carried out by chromatography on an Amberlite XE-64 column in the acid form (170 × 2.5 cm.) as follows. 2 gm. of crude material dissolved in 10 ml. of water (pH 5.2) were added to the top of the column, and elution was carried out with water, a flow rate of 0.67 ml. per minute being used. Sixty fractions of 10 ml. each were collected. Tubes 26 to 30 contained α-aminomalonic acid and a brown material which did not give a ninhydrin reaction. Tubes 31 to 34 apparently contained only α-aminomalonamic acid. Tubes 35 to 37 did not give a ninhydrin

reaction, Tubes 38 to 45 contained glycine, and glycine amide appeared in later fractions. The fractions containing the product were combined and lyophilized. 850 mg. of a light white powder were obtained, which gave the following analyses. Calculated for $C_3H_6O_3N_2$: C 30.5, H 5.1, N 23.7; found, C 30.1, H 5.3, N 23.7. The product was stable in aqueous solution at room temperature for only a few hours, after which time glycine amide formation was detected. When such solutions were heated at 100° for 2 hours, complete destruction of the product with conversion to glycine amide, glycine, and ammonia was observed. Treatment with 2 N hydrochloric acid at 100° for 2 hours yielded glycine and stoichiometric quantities of ammonia.

Procedures—In the course of purification of the *Escherichia coli* glutaminase, assays were performed as follows: Enzyme solution (0.25 ml.) was mixed with 0.25 ml. of freshly prepared 0.08 M L-glutamine (in 0.1 M sodium acetate buffer of pH 4.9) and incubated for 5 to 60 minutes at 37°. The reaction was stopped by addition of 0.5 ml. of 15 per cent trichloroacetic acid, and ammonia was determined after aeration in the presence of potassium carbonate into sulfuric acid traps (25). The assay system used for following the preparation of asparaginase consisted of 0.5 ml. of 0.04 M L-asparagine, 0.5 ml. of enzyme solution, and 0.5 ml. of 0.1 M sodium borate buffer of pH 8.5. With both systems, controls were employed in which enzyme and substrate were separately omitted.

The composition of the reaction mixtures used in the other studies is given in the text. Hydroxylamine hydrochloride solutions were adjusted to the desired pH with sodium hydroxide just prior to use. Hydroxamic acid formation was determined by the method of Lipmann and Tuttle (26). 1 ml. of the reaction mixture was treated with 1.5 ml. of a solution containing 0.67 N hydrochloric acid, 0.37 M ferric chloride, and 0.2 M trichloroacetic acid. After centrifugation, the colors were compared with those of standard solutions in a Klett-Summerson colorimeter equipped with a No. 540 filter. Freshly prepared aqueous solutions of γ-glutamylhydroxamic, β-aspartylhydroxamic, and succinylmonohydroxamic acids were employed as standards. Glutarylmonohydroxamic acid solutions were prepared by dissolving glutaric anhydride in a large excess of hydroxylamine at pH 7 (26). The relative color values for the hydroxamic acids corresponding to glutamine, asparagine, glutaramic acid, and succinamic acid were 100, 67.0, 115, and 115, respectively. In all experiments, controls in which enzyme was omitted were carried out.

Paper chromatography was carried out on Whatman No. 4 paper by an ascending technique and the following solvents: (*a*) formic acid-water-tertiary butanol (15:15:70); (*b*) phenol saturated with 10 per cent aqueous sodium carbonate; and (*c*) *n*-butanol-ethanol-water (2:1:1). Amino

acids were identified by dipping the dried chromatograms in acetone containing 0.25 per cent ninhydrin. For identification of hydroxamic acids the dried chromatograms were dipped in a solution of 95 per cent ethanol containing 1.0 N hydrochloric acid and 0.185 M ferric chloride. The R_F values for β-aspartylhydroxamic, γ-glutamylhydroxamic, succinylmonohydroxamic, and glutarylmonohydroxamic acids were, respectively, as follows: solvent (*a*) 0.15 to 0.24, 0.23 to 0.28, 0.52 to 0.57, 0.58 to 0.66; solvent (*b*) 0.15 to 0.22, 0.28 to 0.35, 0.45 to 0.50, 0.53 to 0.63; solvent (*c*), 0.25 to 0.32, 0.32 to 0.37, 0.54 to 0.59, 0.60 to 0.68.

Succinic, succinamic, glutaric, glutaramic, and pyrrolidonecarboxylic acids were identified on the chromatograms by application of the method of Rydon and Smith (27). In our hands, the procedure gave satisfactory results when the following conditions were employed. After chromatography, the papers were dried for 10 to 20 minutes in a stream of warm air and then placed in an autoclave at 20 pounds pressure for 15 minutes. The autoclave was exhausted rapidly and steam was allowed to flow through the outer jacket for 10 minutes. The papers were exposed to an atmosphere of chlorine for 10 minutes, after which they were placed in a stream of air at room temperature for 30 minutes. The spots were visualized by spraying lightly and evenly with a fine mist of 1 per cent soluble starch in 1 per cent potassium iodide solution. The R_F values for succinamic, succinic, glutaramic, glutaric, and pyrrolidonecarboxylic acids were, respectively, as follows: solvent (*a*), 0.53 to 0.60, 0.70 to 0.74, 0.68 to 0.72, 0.80 to 0.85, 0.62 to 0.70; solvent (*c*), 0.52 to 0.58, 0.67 to 0.71, 0.62 to 0.66, 0.72 to 0.78, 0.40 to 0.45.

Preparation of Purified Glutaminase from E. coli—Lyophilized *E. coli* cells (strain W) were prepared, ground, and extracted as previously described (28). The cell-free extract contained 60 to 80 per cent of the original activity of the cells, and the specific activity was twice that of the cells. The extract obtained from 5 gm. of cells was mixed with the residue obtained by centrifuging 100 ml. of a solution of calcium phosphate gel (dry weight, 15 mg. per ml.). The mixture was mechanically shaken at room temperature for 30 minutes and centrifuged. The enzyme was eluted by shaking the gel residue with 40 ml. of 0.1 M potassium dihydrogen phosphate (pH 4.5) for 20 minutes at 5°, followed by centrifugation at this temperature. The elution procedure was repeated twice, and the eluates were combined and lyophilized. The lyophilized material represented between 20 and 40 per cent of the original activity and exhibited a 15- to 20-fold increase in specific activity over the cell-free extract. The preparation at this stage was free of glutamic decarboxylase and asparaginase activities. It was found possible to double the specific activity at the expense of about half of the total activity by fractionation with sodium sul-

fate as follows. 50 mg. of the lyophilized powder were suspended in 5 ml. of saturated sodium sulfate solution, shaken for 20 minutes at room temperature, and centrifuged. The pellet was dissolved in 1 ml. of water and the solution was stored at 5°. In contrast to the lyophilized powder obtained in the previous step, the enzyme in this form was unstable and lost considerable activity after several days. Such preparations catalyzed the hydrolysis of 10 to 15 mmoles of L-glutamine per mg. of enzyme nitrogen per hour, representing a 50- to 75-fold increase in specific activity over the original cell suspension.

Preparation of Guinea Pig Serum Asparaginase—100 ml. of fresh guinea pig serum were mixed with an equal volume of 30 per cent sodium sulfate. After standing at room temperature for 30 minutes, the mixture was centrifuged at 20°. The pellet was dissolved in 50 ml. of cold water and mixed with the residue obtained by centrifuging an equal volume of calcium phosphate gel. After shaking for 30 minutes, the gel was removed by centrifugation, and the supernatant fluid, containing the enzyme, was stored in the frozen state. Such preparations hydrolyzed 120 to 160 μmoles of L-asparagine per mg. of protein nitrogen per hour, compared to values of 15 to 22 obtained with the original serum.

Preparation of Rat Liver Amidase (Fraction K)—This was prepared as described previously (17). In most preparations the most active fraction was obtained by elution from calcium phosphate gel at pH 6.5. However, with some batches of gel the most active fraction was obtained by elution at pH 5.5 or at pH 6.0. Because of the variability in the behavior of the gel, all of the eluates were assayed and the most active fraction was chosen for study.

Results

Non-Enzymatic Hydroxamic Acid Formation—Non-enzymatic hydroxamic acid formation from amides was not appreciable except with relatively high (0.5 to 1.0 M) concentrations of hydroxylamine. Even with high hydroxylamine concentrations, the rates of non-enzymatic hydroxamic acid formation were considerably lower than those observed with enzyme. The values reported here for enzymatic hydroxamic acid formation were corrected by subtraction of the values observed without enzyme. The effect of pH on non-enzymatic hydroxamic acid formation with 1 M hydroxylamine is described in Fig. 1. All of the amides exhibited maximal non-enzymatic hydroxamic acid formation at pH 6. The curve for asparagine is unique in that it shows a second rise in the region of pH 8.5 to 10. It is also interesting to note that succinamic, glutaramic, and adipamic acids reacted more rapidly than did the respective amino acids.

Although the corresponding dicarboxylic acids did not react non-enzy-

matically with hydroxylamine under the conditions described in Fig. 1, hydroxamic acid formation was observed at a higher temperature. Thus, when a solution of 1 ml. containing 1 mmole of hydroxylamine and 200 μmoles of succinic acid at pH 6 was placed in an autoclave at 15 pounds pressure and 120° for 30 minutes, 40 μmoles of succinylmonohydroxamic acid were formed. Under these conditions, glutaric, glutamic, and aspartic

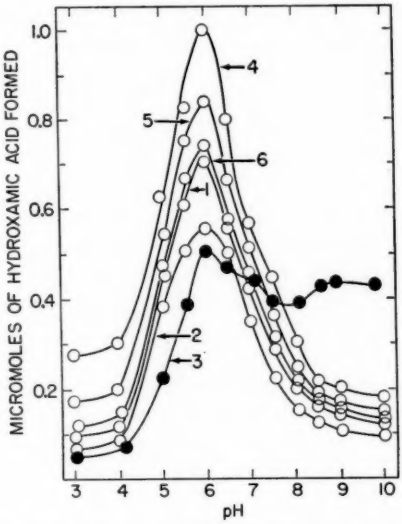


FIG. 1. pH-dependence of the non-enzymatic formation of hydroxamic acids from glutamine (Curve 1), homoglutamine (Curve 2), asparagine (Curve 3), succinamic acid (Curve 4), glutaramic acid (Curve 5), and adipamic acid (Curve 6). The reaction mixtures contained 20 μmoles of amide and 1 mmole of hydroxylamine, adjusted to the appropriate value of pH with sodium hydroxide, in a final volume of 1 ml. The values are expressed as micromoles of hydroxamic acid formed per hour at 37°. The values for adipamic acid and homoglutamine are calculated as glutarylmonohydroxamic and γ-glutamylhydroxamic acids, respectively.

acids yielded 31.4, 4.2, and 3.7 μmoles of hydroxamic acid, respectively. The paper chromatographic behavior of the respective hydroxamic acids formed non-enzymatically from the amides and from the dicarboxylic acids was identical with that of the authentic compounds and with that of the enzymatically formed hydroxamic acids.

Substrate Specificity—Table I describes the susceptibility of a number of amides to hydrolysis by the glutaminase, asparaginase, and rat liver amidase preparations. The glutaminase and asparaginase preparations exhibited a relatively high degree of specificity. Of the amides studied,

TABLE I

*Specificity of Several ω-Amidase Preparations**

Amide	Glutaminase (*E. coli*)	Asparaginase (guinea pig serum)	Rat liver homogenate†	Purified ω-amidase (rat liver)
Acetamide	0	0	0	0
Propionamide	0	0	0	0
Butyramide	0	0	0	0
Isobutyramide	0	0	0	0
Valeramide	0	0	7.1	1.2
Isovaleramide	0	0	0	0
Caproamide	0	0	20.1	6.9
Oxamate	0	0	0	0
Malonamate	0	0	0	0
Succinamate	0	0	29.0	32.0 (75.0)‡
Glutaramate	0	0	39.6	41.0 (92.5)‡
Adipamate	0	0	0	0
α-Ketosuccinamate	0	0	25.0	23.1
α-Ketoglutaramate	0	0	(100)	(100)
α-Ketoadipamate	0	0	0	0
α-Keto-*dl*-γ-methylglutaramate	0	0	0	0
dl-α-Aminomalonamate	0	0	0	0
L-Asparagine	0	(100)	22.2	0
D-Asparagine	0	3.0	0	0
L-Glutamine	(100)	0	10.1	0
D-Glutamine	0.3	0	0	0
L-Homoglutamine	0	0	0	0
D-Homoglutamine	0	0	0	0
α-Methyl-DL-asparagine	0	0	0	0
dl-γ-Methyl-L-glutamine	1.1	0	0	0
α-Methyl-DL-glutamine	25.0	0	0	0
dl-β-Methyl-DL-glutamine	0	0	0	0
L-Isoglutamine	0	0	22.6	2.1
L-Isoasparagine	0	0	9.4	0.5
L-Leucine amide	0	5.3	240	11.5
DL-Proline "	0	0	0	0
DL-Alanine "	0	0	25.0	0
L-Phenylalanine amide	0	8.2	203	2.5
L-Tyrosine amide	0	8.5	140	0

* The reaction mixtures contained 20 μmoles of amide (40 μmoles for the DL compounds), enzyme preparation, and buffer in a final volume of 1.5 ml., and were incubated at 37°. The values given for the purified enzyme preparations are based on ammonia formation (after subtraction of blanks), and are expressed in terms of the most active substrate, arbitrarily assigned a value of 100. Values of less than 0.5 (or 0.1 for the *E. coli* enzyme) are designated as zero. The mixtures were buffered at pH 4.9 (0.05 M sodium acetate), 8.5 (0.05 M sodium borate), and 9.0 (0.05 M sodium borate) for the *E. coli* glutaminase, asparaginase, and rat liver preparations, respectively.

† Fresh rat liver was minced and homogenized with 3 volumes of cold water in a Waring blendor, after which the homogenate was diluted with 3 volumes of 0.1 M sodium borate of pH 9.

‡ At pH 7.0 (0.1 M potassium phosphate buffer).

only glutamine and α-methylglutamine were attacked at an appreciable rate by the *E. coli* glutaminase. The asparaginase preparation hydrolyzed several amino acid α-amides and D-asparagine at low rates. Neither the asparaginase nor the glutaminase preparation hydrolyzed the next lower homologue of asparagine, α-aminomalonamic acid. The low but definite susceptibility of the D isomers of asparagine and glutamine recorded in Table I cannot be ascribed to L isomer impurities in the substrate preparation, since these products were found to contain less than 0.2 per cent of their enantiomorphs by enzymatic tests (29). Furthermore, when large amounts of enzyme were used, the D-amides were hydrolyzed to more than 50 per cent of completion. It is of interest to note that the presence of a D-specific asparaginase has been reported in certain microorganisms (30).

The rat liver homogenate hydrolyzed α-ketoglutaramate, α-ketosuccinamate, succinamate, glutaramate, valeramide, caproamide, glutamine, asparagine, and several amino acid α-amides. The purified liver ω-amidase fraction hydrolyzed the first four of these at approximately the same relative rates, while activity toward the other amides was considerably lower or absent. For example, phenylalanine amide, which was hydrolyzed more than twice as rapidly by the homogenate as was α-ketoglutaramate, was hydrolyzed at less than 12 per cent of the rate observed with α-ketoglutaramate by the purified ω-amidase. The values for the other α-amides were much lower with the purified liver ω-amidase, and there was no activity toward glutamine or asparagine. These data suggest the existence of a hepatic enzyme (or enzymes) different from the amino acid α-amidases and from the amino acid ω-amidases. It is of interest that neither the purified ω-amidase nor the liver homogenate hydrolyzed malonamate, oxamate, or adipamate. Although introduction of an α-keto group in the substrates of susceptible carbon chain length did not lead to inactivity, the presence of a γ-methyl group apparently prevented hydrolysis. A series of homologous diamides from oxamide to adipamide was examined and found to be resistant to deamidation by all of the enzyme preparations.

The initial studies with the liver preparations were carried out at pH 9, since this value of pH was previously found to be optimal for the hydrolysis of α-ketoglutaramate and α-ketosuccinamate (17). The pH optimum for the hydrolysis of L-leucine amide by rat liver extracts has also been reported to be in the neighborhood of 9 (31). However, experiments with glutaramate and succinamate revealed a relatively broad optimal pH range from 6.5 to 7.5; the rates of hydrolysis of these substrates at pH 7.0 are therefore included in Table I. The formation of succinic and glutaric acids as products of enzymatic hydrolysis of the respective amides was demonstrated by paper chromatography. Oxalacetic and α-ketoglutaric acids were found previously to be products of hydrolysis of α-ketosuccinamic and α-ketoglutaramic acids (17).

XUM

Synthesis of Hydroxamic Acids by Purified Liver ω-Amidase Preparation—Enzyme systems which catalyze the exchange of the amide group of glutamine and asparagine with hydroxylamine and certain other amines have been found in a variety of species (11–15, 32–34), and hydroxamic acid formation has also been observed with a number of peptidase preparations (35–39). In the present investigation it was found that the liver ω-amidase preparation catalyzed the formation of the corresponding hydroxamic acids

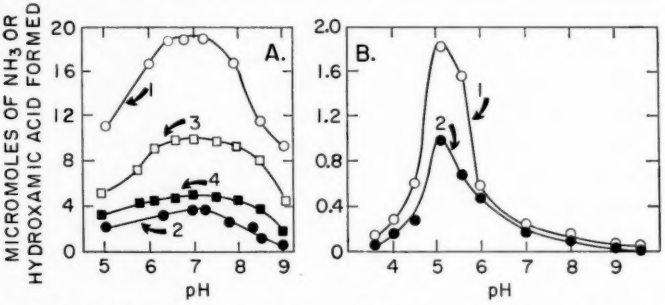


FIG. 2. *A*, pH-dependence of the enzymatic hydrolysis of glutaramic and succinamic acids and of the analogous transfer reactions. Curves 1 and 2, formation of hydroxamic acid and ammonia, respectively, from succinamic acid; Curves 3 and 4, formation of hydroxamic acid and ammonia, respectively, from glutaramic acid. The reaction mixtures contained 10 μmoles of amide, 40 γ of liver ω-amidase N, 25 μmoles of hydroxylamine (Curves 1 and 3), and buffer in a final volume of 1 ml. 0.05 M sodium acetate (pH 4.9 to 6.5), 0.05 M potassium phosphate (pH 6.8 to 7.6), and 0.05 M sodium borate (pH 7.7 to 9.1) buffers were used. The mixtures were incubated at 37° for 10 minutes (Curves 1 and 3) or 60 minutes (Curves 2 and 4), and the values are expressed as micromoles of ammonia or hydroxamic acid formed per hour. *B*, pH-dependence of the formation of hydroxamic acid from glutaric and succinic acids. Curve 1, glutaric acid; Curve 2, succinic acid. The reaction mixtures contained 200 μmoles of dicarboxylic acid, 1 mmole of hydroxylamine (adjusted to the appropriate value of pH), and 200 γ of liver ω-amidase N in a final volume of 1 ml. The incubation time was 30 to 60 minutes at 37°; the values are expressed as in *A*.

from succinamic and glutaramic acids and hydroxylamine. These were identified by paper chromatography as described above. The effect of pH on the ability of the liver ω-amidase to catalyze the hydrolysis of glutaramic and succinamic acids, and to catalyze the formation of hydroxamic acids from these amides in the presence of hydroxylamine, is described in Fig. 2, *A*. All of these reactions proceeded most rapidly at about pH 6.5 to 7.5. Under these conditions, the formation of succinylmonohydroxamic acid occurred about 6 times more rapidly than did the hydrolysis of succinamic acid. With glutaramic acid, the rate of hydroxamic acid formation was about twice that of hydrolysis. Although succinamic acid

was less susceptible than glutaramic acid to the hydrolytic activity of the enzyme preparation, succinamic acid formed a hydroxamic acid more rapidly than did glutaramic acid.

The time-course of the deamidation of succinamic acid in the presence and absence of hydroxylamine is described in Fig. 3. As would be expected from the data given in Fig. 2, deamidation proceeded more rapidly in the presence of hydroxylamine. The formation of hydroxamic acid leveled off at about 85 per cent of completion, indicating that about 15 per cent of the substrate was hydrolyzed. Similar results were observed with glutaramic acid; in these experiments 70 per cent of the substrate was converted to glutarylmonohydroxamic acid.

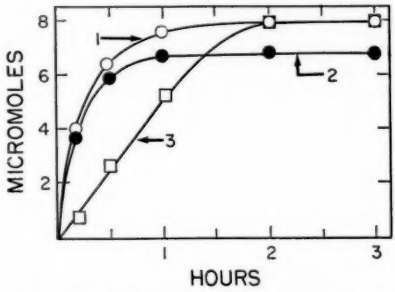


FIG. 3. Time-course of the deamidation of succinamic acid in the presence and absence of hydroxylamine. Curves 1 and 2, ammonia and hydroxamic acid formation, respectively, in the presence of hydroxylamine; Curve 3, deamidation in the absence of hydroxylamine. The reaction mixtures contained 8 μmoles of succinamic acid, 25 μmoles of hydroxylamine (Curves 1 and 2), and 40 γ of liver ω-amidase N in 1 ml. of 0.05 M potassium phosphate buffer at pH 7.0; 37°.

The formation of hydroxamic acid with the ω-amidase preparation was not dependent upon the presence of cofactors such as magnesium or manganous ions, adenosine triphosphate, adenosine diphosphate, or inorganic phosphate; nor were these reactions accelerated by arsenate. No hydroxamic acid was formed with those amides which were poor substrates for hydrolysis or those which were not hydrolyzed, such as malonamate, adipamate, caproamide, leucine amide, asparagine, or glutamine.

Incubation of glutaric or succinic acids with hydroxylamine and the ω-amidase at pH 7 did not lead to significant hydroxamic acid formation. However, when high concentrations of these acids and of hydroxylamine were incubated with the enzyme at pH 5, measurable quantities of the corresponding hydroxamic acids were formed (Fig. 2, *B*). These were identified by paper chromatography. The rate of hydroxamic acid formation from succinic and glutaric acids was considerably less rapid than the for-

XUM

mation of the hydroxamic acids from the respective amides, and the reaction did not proceed beyond about 5 per cent of completion, even after 6 hours of incubation. Furthermore, hydroxamic acid formation decreased appreciably when the concentration of either hydroxylamine or dicarboxylic acid was reduced (Table II). Under these conditions, no hydroxamic acid was formed from oxalic, malonic, adipic, glutamic, or aspartic acids.

TABLE II

*Synthesis of Hydroxamic Acids from Dicarboxylic Acids**

Enzyme preparation	pH	Hydroxylamine concentration	Substrate		Hydroxamic acid formed
		M		μmoles	μmoles per hr.
Liver ω-amidase	5.0	0.4	Glutaric acid	200	1.91
" "	5.0	0.4	" "	100	1.20
" "	5.0	0.4	" "	50	0.82
" "	5.0	0.2	" "	200	1.32
" "	5.0	0.1	" "	200	1.00
" "	5.0	0.05	" "	200	0.74
Glutaminase	5.0	1.0	L-Glutamic acid	100	5.91
"	5.0	1.0	" "	50	5.04
"	5.0	1.0	" "	25	3.00
"	5.0	0.5	" "	100	4.55
"	5.0	0.0625	" "	100	0.59
"	5.0	1.0	D-Glutamic "	200	0
Asparaginase	7.0	1.0	L-Aspartic "	200	1.50
"	7.0	1.0	" "	100	1.06
"	7.0	1.0	" "	50	0.77
"	7.0	0.5	" "	200	0.92
"	7.0	0.1	" "	200	0.15
"	7.0	1.0	D-Aspartic "	200	0

* The experimental conditions were as described in Figs. 2 and 3, except that 200 γ of asparaginase N were used, and as noted in the table.

Synthesis of Hydroxamic Acids with Glutaminase and Asparaginase Preparations—An investigation of the *E. coli* glutaminase and guinea pig serum asparaginase preparations revealed that these preparations could also catalyze hydroxylamine transfer reactions (Fig. 4). With both enzymes, the transfer activity was considerably lower than the hydrolytic activity. With the asparagine system (Fig. 4, *B*), maximal transfer activity was observed at pH 5.9, compared to an optimal pH range of about 7.5 to 8.5 for hydrolysis of asparagine. Neither the hydrolysis nor the transfer reactions catalyzed by the glutaminase and asparaginase preparations were accelerated by manganous or magnesium ion, adenosine nucleotides, phosphate, or arsenate.

Formation of hydroxamic acid from glutamic acid and hydroxylamine was observed with the *E. coli* glutaminase preparation (Fig. 4, *A*).[4] The pH optimum for this reaction was about 5 compared to 5.8 for the formation of hydroxamic acid from glutamine, although the rates of these reac-

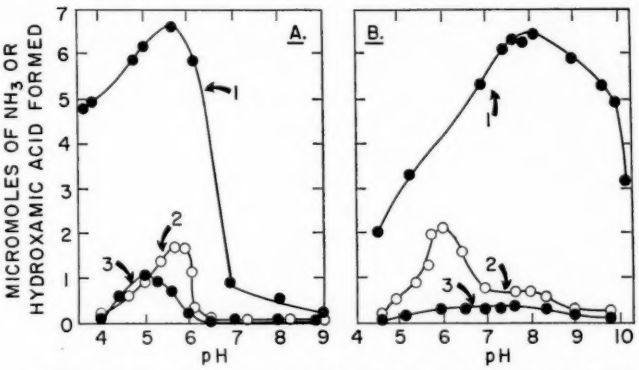


FIG. 4. pH-dependence of the enzymatic hydrolysis of glutamine and asparagine, of the analogous transfer reactions, and of the formation of hydroxamic acids from glutamic and aspartic acids. *A*, *E. coli* glutaminase; Curve 1, formation of NH_3; Curve 2, formation of hydroxamic acid from glutamine; Curve 3, formation of hydroxamic acid from glutamic acid. The reaction mixtures contained 20 μmoles of L-glutamine or L-glutamic acid, 3 γ of enzyme N, and (Curves 2 and 3) 1 mmole of hydroxylamine in final volume of 1 ml. For Curve 1, 0.05 M sodium acetate (pH 3.6 to 5.8), 0.05 M potassium phosphate (pH 6.1 to 6.9), and 0.05 M sodium borate (pH 7.2 to 9.0) buffers were used. In Curves 2 and 3, the hydroxylamine solution was adjusted to the appropriate pH. The mixtures were incubated at 37° for 15 minutes. *B*, guinea pig serum asparaginase; Curve 1, formation of NH_3; Curve 2, formation of hydroxamic acid from asparagine; Curve 3, formation of hydroxamic acid from aspartic acid. The reaction mixtures contained 20 μmoles of L-asparagine or 200 μmoles of L-aspartic acid, 50 γ of enzyme N, and (Curves 2 and 3) 1 mmole of hydroxylamine in a final volume of 1 ml. For Curve 1, 0.05 M sodium acetate (pH 4.4 to 5.2) and 0.05 M sodium borate (pH 6.9 to 10.1) buffers were used. In Curves 2 and 3 the hydroxylamine solution was adjusted to the appropriate pH. The mixtures were incubated at 37° for 30 to 120 minutes. The values are expressed as micromoles per hour.

tions were of the same order of magnitude. On the other hand, the formation of β-aspartylhydroxamic acid from aspartic acid and hydroxylamine with the guinea pig serum asparaginase was considerably less rapid than the comparable reaction with asparagine. β-Aspartylhydroxamic

[4] The hydroxamic acid was identified by paper chromatography. Chromatograms of γ-glutamylhydroxamic acid also showed the presence of small amounts of pyrrolidonecarboxylic acid. The tendency for ring closure to occur is about 20 times greater with γ-glutamylhydroxamic acid than with glutamine.

acid formation from aspartic acid exhibited a broad optimal pH range and was dependent upon a relatively high aspartic acid concentration. Miller and Waelsch have observed a similar reaction with extracts of *Proteus vulgaris* (40). The dependence of these reactions on high substrate and hydroxylamine concentrations is similar to that observed in experiments with succinic and glutaric acids and the liver ω-amidase preparation. After prolonged incubation, these reactions proceeded to about 5 per cent of completion.

Enzymatic Hydrolysis of Hydroxamic Acids—Experiments with the hydroxamic acids corresponding to glutamine, asparagine, and succinamic acid revealed that the hydroxamic acids were hydrolyzed much more slowly by the respective enzyme preparations than were the amides (*cf.* (11)). In these studies the disappearance of hydroxamic acid was determined quantitatively by the ferric chloride procedure, and the formation of the corresponding dicarboxylic acids was observed by paper chromatography. L-γ-Glutamylhydroxamic acid was hydrolyzed by the glutaminase preparation at about 10 per cent of the rate observed with L-glutamine. The pH optimum was 5.0, and the reaction appeared to proceed to completion. However, there was about a 10 per cent non-enzymatic disappearance of hydroxamic acid under these conditions. Hydrolysis of L-β-aspartylhydroxamic acid by the asparaginase preparation occurred most rapidly at pH 8.0. At this pH value the reaction proceeded virtually to completion at about 15 per cent of the rate observed with L-asparagine. Succinylmonohydroxamic acid was hydrolyzed by the liver ω-amidase preparation at about 1.5 per cent of the rate found with succinamic acid; the pH-activity curves for hydrolysis of these compounds were similar and, in the presence of sufficient enzyme, the reaction went to completion.

Effect of Hydroxylamine Concentration on Hydroxamic Acid Formation from Amides—In addition to the marked difference in the relative rates of hydrolysis and transfer between the glutaminase and asparaginase preparations, on the one hand, and the liver ω-amidase on the other, it was observed that the former systems required considerably higher concentrations of hydroxylamine for optimal activity than the latter. As described in Fig. 5, maximal activity of the ω-amidase preparation was achieved with 0.05 M hydroxylamine, while 12 and 20 times as much amine was required for the glutaminase and asparaginase systems, respectively. The relatively high affinity of the liver ω-amidase for hydroxylamine is also indicated by the fact that the reaction proceeded rapidly with stoichiometric quantities of hydroxylamine and amide.

Studies with Other Substrates—Attempts to evaluate the formation of hydroxamic acid from α-ketosuccinamic acid with the liver ω-amidase were unsuccessful, due to the fact that the keto acid itself gave a color with ferric

chloride. On the other hand, α-ketoglutaramate appeared to be active with hydroxylamine, as judged by the increase in color on treatment with ferric chloride after incubation of the keto acid with hydroxylamine and liver ω-amidase. In a typical experiment with 20 μmoles of α-ketoglutaramate, 20 μmoles of hydroxylamine, and 350 γ of enzyme N at pH 9.0 in a final volume of 1.0 ml., 0.85 μmole of hydroxamic acid (calculated as glutarylmonohydroxamic acid) was formed per hour. The pH optimum for this reaction was 8.5 to 9.0, or close to that for hydrolysis of α-ketoglutaramate (17). With higher concentrations of hydroxylamine or α-ketoglutaramate, an increase in hydroxamic acid formation was observed. Thus, under the above conditions, the hydroxamate formation was increased by 8-fold when 400 μmoles of hydroxylamine were used, and by about 4-fold when the concentrations of both amide and amine were doubled.

No detectable hydrolysis of L-aspartic acid β-ethyl ester by the asparaginase preparation was observed, and enzymatic hydroxamic acid formation from this compound could not be detected. On the other hand, the glutaminase preparation hydrolyzed L-glutamic acid γ-ethyl ester at about 0.1 per cent of the rate with L-glutamine, and catalyzed γ-glutamylhydroxamic acid formation at approximately 4 times the non-enzymatic rate at pH 5. The susceptibility of esters to attack by peptidases has been extensively considered by Neurath and Schwert (41).

DISCUSSION

Each of the enzyme preparations studied here has been found to catalyze four reactions:

$$RCONH_2 + H_2O \rightarrow RCOOH + NH_3 \quad (1)$$

$$RCONH_2 + NH_2OH \rightarrow RCONHOH + NH_3 \quad (2)$$

$$RCONHOH + H_2O \rightarrow RCOOH + NH_2OH \quad (3)$$

$$RCOOH + NH_2OH \rightarrow RCONHOH + H_2O \quad (4)$$

With all of the enzyme preparations, Reaction 3 proceeded more slowly than did Reaction 1, and Reaction 4 always took place at a very low rate compared to that of Reaction 1.

A finding of considerable interest was that, with succinamic and glutaramic acids, the liver ω-amidase catalyzed Reaction 2 at a more rapid rate than Reaction 1. Furthermore, a relatively low hydroxylamine concentration was required (Fig. 5). Although hydrolysis of glutaramic acid was faster than succinamic acid, the latter substrate reacted more rapidly with hydroxylamine than did the former (Fig. 2). The non-enzymatic formation of succinylmonohydroxamic acid was also more rapid than that of glutarylmonohydroxamic acid (Fig. 1). In contrast to the liver ω-ami-

XUM

dase, glutaminase and asparaginase catalyzed Reaction 1 much more rapidly than Reaction 2, and much higher concentrations of hydroxylamine were required for maximal transfer activity.

The pH optima for hydrolysis and hydroxamic acid formation from the amide were about the same with the liver ω-amidase (pH 7.0) and glutaminase (pH 5.8) preparations, while the pH optimum for hydroxamic acid formation from asparagine was 5.9, compared to an optimum of 8.0 for hydrolysis of asparagine and β-aspartylhydroxamic acid. On the other hand, Reaction 4 usually had an optimal pH value which was more acid than the optimal pH for Reactions 1, 3, and (except for asparaginase) 2.

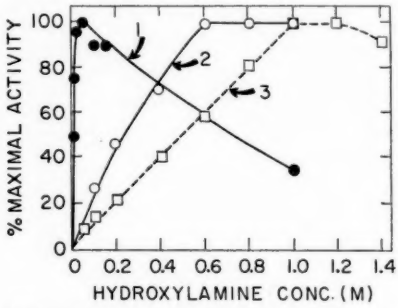


FIG. 5. Effect of hydroxylamine concentration on the rate of the transfer reactions. Curve 1, liver ω-amidase preparation with glutaramate; pH 7.0. Curve 2, *E. coli* glutaminase with L-glutamine; pH 5.7. Curve 3, guinea pig serum asparaginase with L-asparagine; pH 5.8. The reaction mixtures contained 25 μmoles of amide, hydroxylamine in the indicated concentrations, and sufficient enzyme to yield 2 to 3 μmoles of hydroxamic acid (at optimal hydroxylamine concentration) within the experimental period (15 or 30 minutes).

In recent years, a number of hydrolytic enzymes have been found to catalyze transfer reactions. These include certain phosphatases, nucleotidases, glycosidases (see, for example (42–45)), as well as enzymes which act on amide and peptide bonds (11–16, 32–40, 46). It has been suggested that such transfer and hydrolysis reactions are probably catalyzed by the same enzyme (46–48). Evidence consistent with this concept, as applied to the enzymes studied here, includes the observation that the relative rates of hydroxamic acid formation and hydrolysis were the same (for each enzyme) with the original crude preparations as with the final preparations. Furthermore, with each enzyme, the substrate specificity for each of the reactions was the same. However, if a single enzyme does catalyze both hydrolysis and transfer reactions, then an explanation must be sought for the markedly different relative reaction rates and pH optima.

Fruton and collaborators (35–39, 47), in an extensive study of transpeptidation replacement reactions catalyzed by hydrolytic enzymes (*e.g.*, papain, ficin, cathepsin C), observed that, in general, optimal hydrolysis occurred at about pH 5, while transpeptidation was favored by more alkaline values of pH. In our experiments, the optimal pH for hydroxamic acid formation from the amide was the same or lower than the pH optimum for hydrolysis. It is probable that the pH optimum for a transfer reaction may be influenced by at least several factors, including the pK values of the base and the cation, other properties of the substrate, and the characteristics of the enzyme itself. A comparison of the pH-activity curves for the nonenzymatic formation of hydroxamic acids from glutamine and asparagine (Fig. 1) is of interest in this connection.

Assuming that the hydrolysis and transfer reactions are catalyzed by the same enzyme, the results may be explained, in analogy with Fruton's formulation, in terms of an enzyme-amide complex which may react either with water or with hydroxylamine. That this cannot be a simple competitive phenomenon is evident from the fact that appreciable hydroxamic acid is formed with a concentration of hydroxylamine which is very low compared to that of water. Furthermore, the preferential reaction of hydroxylamine can probably not be attributed entirely to participation of an enzyme, since similar reactions occur non-enzymatically. The enzymatic synthesis of a hydroxamic acid from hydroxylamine and a dicarboxylic acid (*e.g.*, succinic, glutamic), as in Reaction 4, may be considered formally as the reversal of the hydrolysis reaction (Reaction 3). The very low rate and extent of this reaction are compatible with such an interpretation. One may also consider this phenomenon in terms of an enzyme-dicarboxylic acid complex which may react either with water or with hydroxylamine. The small extent of such reactions may be ascribed to a low affinity of the enzyme for the free acid as compared to the amide; this concept is consistent with the requirement for a relatively high substrate concentration. It is of interest that these reactions proceeded at higher temperature without enzyme.

The transfer reactions described here differ from that reported to occur with glutamine in a number of plant, animal, and bacterial systems, in which adenosine nucleotides, phosphate, and manganese or magnesium ions are required. The latter reaction appears to be associated with the glutamine synthesis system, and attempts in several laboratories to separate the glutamine synthesis activity from this transfer activity have not been successful (49, 50, 10). The glutamine transfer activity studied here also differs from that of certain transfer systems studied by Waelsch (12) in which the glutaminase activity was found to be relatively low. We have also found that a fraction obtained from a glutamine-requiring mutant of

XUM

Aerobacter aerogenes (51) catalyzes γ-glutamylhydroxamic acid formation from glutamine much more rapidly than it hydrolyzes glutamine.[5] No cofactors are needed, and optimal activity is achieved with a low (0.01 M) hydroxylamine concentration. On the other hand, in studies with a purified preparation of the phosphate-activated glutaminase of rat liver (5),[6] we have been unable to detect hydroxamic acid formation. There are evidently a number of enzymes which catalyze hydroxamic acid formation from glutamine. At this time it would appear that enzyme preparations which exhibit such activity also catalyze (*a*) hydrolysis of glutamine or γ-glutamyl peptides (12, 16) or (*b*) synthesis of glutamine. It may be postulated that an activated enzyme-substrate complex is formed in both types of reactions, and that hydroxylamine can readily react with such an intermediate. The relative rates of hydrolysis and transfer may reflect differences in the nature of the intermediate complex.

The rôle of the liver ω-amidase system in the transamination-deamidation reactions of glutamine and asparagine has been considered previously (52). The present observation that this enzyme can catalyze exchange reactions leading to hydroxamic acid formation suggests a possible mechanism for α-ketosuccinamate formation; *i.e.*, by reaction of oxalacetate or a derivative of this keto acid with ammonia or with an amino donor. The question as to whether hydroxylamine serves as a model for a natural substrate must await further study.

SUMMARY

1. A purified rat liver ω-amidase fraction hydrolyzed α-ketoglutaramic, α-ketosuccinamic, glutaramic, and succinamic acids, but did not deamidate glutamine, asparagine, and a number of other amides. The enzyme also catalyzed the formation of succinylmonohydroxamic and glutarylmonohydroxamic acids from the corresponding amides and relatively low concentrations of hydroxylamine; these reactions proceeded much more rapidly than did the analogous hydrolysis reactions. Both hydrolysis and transfer reactions proceeded most rapidly at about pH 7.

2. Preparations of guinea pig serum asparaginase and *Escherichia coli* glutaminase catalyzed hydroxamic acid formation from asparagine and glutamine, respectively, at rates which were low in comparison to those of hydrolysis. In contrast to the liver ω-amidase system, high concentrations of hydroxylamine were required for hydroxamic acid formation. The pH-dependence of these reactions was determined.

3. Succinylmonohydroxamic, β-aspartylhydroxamic, and γ-glutamylhydroxamic acids were hydrolyzed by the respective enzyme preparations at much slower rates than observed with the corresponding amides.

[5] Levintow, L., and Meister, A., unpublished.

[6] Generously provided by Mr. M. C. Otey and Dr. J. P. Greenstein.

4. In the presence of hydroxylamine, all of the enzyme preparations catalyzed hydroxamic acid synthesis from the dicarboxylic acids corresponding to the susceptible amides. This and the other findings are considered in terms of the concept that the transfer, hydrolysis, and synthesis reactions are catalyzed by the same enzyme.

5. The pH-dependence of the non-enzymatic formation of hydroxamic acids from glutamine, homoglutamine, asparagine, succinamic acid, and glutaramic acid, and hydroxylamine at 37°, was studied. Non-enzymatic formation of monohydroxamic acids from aspartic, glutamic, succinic, and glutaric acids was observed at 120°.

6. The preparation of *dl*-α-aminomalonamic acid, *dl*-β-methyl-DL-glutamic acid, *dl*-β-methyl-DL-glutamic acid γ-ethyl ester, and *dl*-β-methyl-DL-glutamine is described.

BIBLIOGRAPHY

1. Krebs, H. A., *Biochem. J.*, **29,** 1951 (1935).
2. Grassmann, W., and Mayr, O., *Z. physiol. Chem.*, **214,** 185 (1933).
3. Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7,** 433 (1947).
4. Errera, M., and Greenstein, J. P., *J. Biol. Chem.*, **178,** 495 (1949).
5. Otey, M. C., Birnbaum, S. M., and Greenstein, J. P., *Arch. Biochem. and Biophys.*, **49,** 245 (1954).
6. Greenstein, J. P., and Carter, C. E., *J. Biol. Chem.*, **165,** 741 (1946).
7. Greenstein, J. P., and Price, V. E., *J. Biol. Chem.*, **178,** 695 (1949).
8. Meister, A., and Tice, S. V., *J. Biol. Chem.*, **187,** 173 (1950).
9. Meister, A., Sober, H. A., Tice, S. V., and Fraser, P. E., *J. Biol. Chem.*, **197,** 319 (1952).
10. Levintow, L., and Meister, A., *J. Biol. Chem.*, **209,** 265 (1954).
11. Grossowicz, N., Wainfan, E., Borek, E., and Waelsch, H., *J. Biol. Chem.*, **187,** 111 (1950).
12. Waelsch, H., *Advances in Enzymol.*, **13,** 237 (1952).
13. Stumpf, P. K., and Loomis, W. D., *Arch. Biochem.*, **25,** 451 (1950).
14. Stumpf, P. K., Loomis, W. D., and Michelson, C., *Arch. Biochem. and Biophys.*, **30,** 126 (1951).
15. Delwiche, C. C., Loomis, W. D., and Stumpf, P. K., *Arch. Biochem. and Biophys.*, **33,** 333 (1951).
16. Williams, W. J., and Thorne, C. B., *J. Biol. Chem.*, **210,** 203 (1954).
17. Meister, A., *J. Biol. Chem.*, **200,** 571 (1953).
18. Meister, A., *J. Biol. Chem.*, **210,** 17 (1954).
19. Fischer, E., and Dilthey, A., *Ber. chem. Ges.*, **35,** 844 (1902).
20. Jeffery, G. H., and Vogel, A. I., *J. Chem. Soc.*, 1101 (1934).
21. Oelkers, L., *Ber. chem. Ges.*, **22,** 1566 (1889).
22. Renfrow, W. B., Jr., and Hauser, C. R., *J. Am. Chem. Soc.*, **59,** 2308 (1937).
23. Snyder, H. R., Shekleton, J. F., and Lewis, C. D., *J. Am. Chem. Soc.*, **67,** 310 (1945).
24. Done, J., and Fowden, L., *Biochem. J.*, **51,** 451 (1952).
25. Greenstein, J. P., and Leuthardt, F., *J. Nat. Cancer Inst.*, **5,** 209 (1944).
26. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
27. Rydon, H. N., and Smith, P. W. G., *Nature*, **169,** 922 (1952).

XUM

28. Rudman, D., and Meister, A., *J. Biol. Chem.*, **200,** 591 (1953).
29. Meister, A., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **192,** 535 (1951).
30. Altenbern, R. A., and Housewright, R. D., *Arch. Biochem. and Biophys.*, **49,** 130 (1954).
31. Errera, M., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **8,** 71 (1947).
32. Waelsch, H., Owades, P., Borek, E., Grossowicz, N., and Schou, M., *Arch. Biochem.*, **27,** 237 (1950).
33. Schou, M., Grossowicz, N., Lajtha, A., and Waelsch, H., *Nature*, **167,** 818 (1951).
34. Lajtha, A., Mela, P., and Waelsch, H., *J. Biol. Chem.*, **205,** 553 (1953).
35. Johnston, R. B., Mycek, M. J., and Fruton, J. S., *J. Biol. Chem.*, **185,** 629 (1950).
36. Johnston, R. B., Mycek, M. J., and Fruton, J. S., *J. Biol. Chem.*, **187,** 205 (1950).
37. Fruton, J. S., Johnston, R. B., and Fried, M., *J. Biol. Chem.*, **190,** 39 (1951).
38. Durell, J., and Fruton, J. S., *J. Biol. Chem.*, **207,** 487 (1954).
39. Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S., *J. Biol. Chem.*, **195,** 645 (1952).
40. Miller, A., and Waelsch, H., *Federation Proc.*, **12,** 246 (1953).
41. Neurath, H., and Schwert, G. W., *Chem. Rev.*, **46,** 69 (1950).
42. Axelrod, B., *J. Biol. Chem.*, **172,** 1 (1948).
43. Hehre, E. J., *J. Biol. Chem.*, **177,** 267 (1949).
44. Meyerhof, O., and Green, H., *J. Biol. Chem.*, **183,** 377 (1950).
45. Heppel, L. A., Whitfeld, P. R., and Markham, R., *Biochem. J.*, **56,** p. iii (1954).
46. Hanes, C. S., Hird, F. J. R., and Isherwood, F. A., *Nature*, **167,** 818 (1951); *Biochem. J.*, **51,** 25 (1952).
47. Fruton, J. S., *Yale J. Biol. and Med.*, **22,** 263 (1950).
48. Hird, F. J. R., and Springell, P. H., *Biochem. J.*, **56,** 417 (1954).
49. Waelsch, H., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **2,** 109 (1952).
50. Elliott, W. H., *J. Biol. Chem.*, **201,** 661 (1953).
51. Davis, B. D., VIth International Congress of Microbiology, Symposia, Rome 23 (1953).
25. Meister, A., and Fraser, P. E., *J. Biol. Chem.*, **210,** 37 (1954).

GLUCOSE CATABOLISM IN LIVER SLICES VIA THE PHOSPHOGLUCONATE OXIDATION PATHWAY*

By BEN BLOOM, FRANK EISENBERG, JR., AND DEWITT STETTEN, JR.

(From the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York, New York, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland)

(Received for publication, October 13, 1954)

In earlier communications (1–3) experiments have been described in which liver slices were incubated with glucose, gluconate, or lactate, labeled in specific positions with C^{14}. The recoveries of isotope in CO_2 and in fatty acids have been measured, and on the basis of these measurements it has been concluded that a pathway other than the Embden-Meyerhof route[1] is operating in this tissue. It has further been estimated (3) that approximately half of the glucose catabolized by rat liver slices enters the Embden-Meyerhof pathway, whereas the remaining half is degraded by steps which resemble those of the phosphogluconate oxidation pathway.

In the present study, in addition to CO_2 and fatty acids, glycogen has been recovered from liver slices after incubation with labeled substrates. The glucose derived from this glycogen has been subjected to chemical degradation (4) to determine the abundance of isotope in certain positions. On the basis of the known reactions which together comprise the phosphogluconate oxidation pathway (5, 6), certain predictions relating to the redistribution of carbon atoms within the sugar molecule can be made, and it was considered of interest to explore the possible occurrence of such redistribution.

EXPERIMENTAL

Isotopic Compounds—The sources or methods of preparation of D-glucose-1-C^{14}, D-glucose-2-C^{14}, D-glucose-6-C^{14}, sodium gluconate-1-C^{14}, and sodium gluconate-6-C^{14} have been described (1, 3). D-Ribose-1-C^{14} was obtained

* At the suggestion of Dr. B. Horecker, this name is given to a pathway which has variously been termed the hexose monophosphate shunt, the Warburg-Dickens-Lipmann pathway, the direct oxidative route.

[1] Whereas the terms glycolysis, glycolytic, glycolyzed, etc., have been used in reports from this laboratory, as well as by others, to designate the Embden-Meyerhof pathway, such usage is incorrect. It would seem advantageous to reserve these terms for lactate production from glucose without implication of mechanism. In the present report, the term Embden-Meyerhof is adhered to for designation of that pathway by which 1 mole of glucose, via glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, etc., can give rise to 2 moles of lactate.

from the National Bureau of Standards. Unless otherwise indicated, all compounds assayed for C^{14} were converted to $BaCO_3$ and counted at infinite thickness (7).

Incubation Procedure—Adult male rats of the Sherman strain, maintained on Purina chow, were used in this study. For the 3 days prior to sacrifice the rats were offered *ad libitum* a diet containing 60 per cent of glucose (1). The animals were sacrificed by a blow on the head, and liver slices were prepared with the Stadie-Riggs microtome (8).

The incubation medium employed was the potassium-rich buffer found to be most favorable for glycogen synthesis by Hastings *et al.* (9). The amount of substrate added to each flask is indicated in Tables I and II. A description of the incubation flask as well as details of the incubation procedure has been reported (1).

Isolations—It was found desirable for isolation of glycogen to terminate the incubation by acidification of the buffer with trichloroacetic acid rather than with mineral acid which was previously used (1). Glycogen was isolated from liver slices by the method of Stetten and Boxer (10). Fatty acids isolated from the combined supernatant fluid and washings of the glycogen precipitation were assayed directly for C^{14} (3). The C^{14} activities of carbons 1 and 6 of glycogen-glucose (*i.e.*, glucose derived from glycogen) were determined by the method of Eisenberg (4).

RESULTS AND DISCUSSION

The recoveries of C^{14} in CO_2 and in fatty acids (Table I) after incubation of rat liver slices with glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, and gluconate-6-C^{14} are in good agreement with values secured earlier in comparable experiments (3).

Probably as a consequence of the direct incorporation of glucose from the medium into glycogen, the specific activity of glycogen is relatively independent of the location of the label in glucose. This is borne out by finding the bulk of the isotope in carbon 1 of glycogen-glucose after incubation of tissue with glucose-1-C^{14}; in carbon 6 after incubation with glucose-6-C^{14}. Carbon 1 of nutrient glucose makes a small contribution to position 6 of glycogen, and likewise carbon 6 of glucose of the medium makes a small contribution to position 1. Such randomization between positions 1 and 6 of glucose, apparently of limited extent under our experimental conditions, may well result from the reversible operation of the Embden-Meyerhof pathway down to the level of the triosephosphate isomerase reaction. This limited extent of randomization between carbons 1 and 6 of glucose has been observed in rats (11), yeast (12), and perfused mammary gland (13).

Incubation of liver slices with glucose-2-C^{14} yielded glycogen which con-

tained about 20 times as much C^{14} in position 1 as in position 6 (Table I), a distribution which cannot be pictured as resulting from the unique operation of the Embden-Meyerhof pathway. This asymmetry might well have resulted, however, from the operation of the phosphogluconate oxidation sequence in which carbon 2 of glucose (Fig. 1, ■C) serves as a precursor for carbons 1 and 3 of regenerated hexose.

The finding of glycogen unsymmetrically labeled in positions 1 and 6 after incubation with glucose-2-C^{14} would appear to conflict with the established finding that symmetrically labeled glucose arises from 2- or

TABLE I

Incorporation of Isotope into Glycogen, Fatty Acids, and CO_2

Liver slices were incubated with 4.5 ml. of buffer containing 50 μmoles of glucose or gluconate. Radiochemical yields are calculated per 500 mg. of tissue for the 2 hour incubation period. Each flask actually contained from 998 to 1010 mg. of tissue and from 1×10^4 to 2×10^4 c.p.m. of substrate C^{14}. The values recorded below have been normalized to 2×10^4 c.p.m. per flask. Three experiments were pooled for all determinations except the CO_2 values, which are the averages of the three experiments.

Substrate	Per cent radiochemical yield		Specific activity* of glycogen carbons			
	CO_2	Fatty acid	1-6 (*a*)	1 (*b*)	6 (*c*)	2-5† (*d*)
Glucose-1-C^{14}	4.74	0.23	1750	7400	215	720
Glucose-2-C^{14}	1.53	0.31	2350	1080	55	3240
Glucose-6-C^{14}	1.46	0.69	2220	94	11,110	530
Gluconate-1-C^{14}	2.64	0	0			
Gluconate-6-C^{14}	0.28	0.19	65	57	242	23

* Counts per minute per milliatom of carbon.

† Calculated from the expression $d = (6a - b - c)/4$.

3-labeled lactic and pyruvic acids (14, 15). In these latter studies, however, the symmetrical synthesis of glucose from triose, via reversal of the Embden-Meyerhof pathway, may well mask the preferential movement of carbon 2 of glucose to position 1.

The distribution of carbon atoms of gluconate in glycogen supports our earlier assumption (3) that gluconic acid, in so far as it is catabolized, enters the phosphogluconate oxidation pathway, presumably after phosphorylation at position 6. As will be seen from Fig. 1, ●C, carbon 1 of gluconate does not, by this pathway, enter directly into any position of regenerated hexose. This carbon atom of gluconate probably labels glucose only incident to CO_2 fixation (16) which, at the level of isotope employed, was not detected.

XUM

In contrast to carbon 1, carbon 6 of gluconate was readily recoverable in glycogen, so distributed that far more isotope was present in carbon 6 of glucose than in carbon 1. These results could not have been due to the direct reduction of gluconate, since this possibility had been ruled out in the experiment with gluconate-1-C^{14}. A mechanism by which gluconate-6-C^{14} could give rise to glucose labeled in position 6 is indicated in Fig. 1, ▲C. Another possibility by which the same end could be achieved is the transfer of "active" glycolaldehyde to tetrose-4-phosphate-4-C^{14} (Fig. 1) under the influence of transketolase (17, 18). Labeling of carbon 1 of glu-

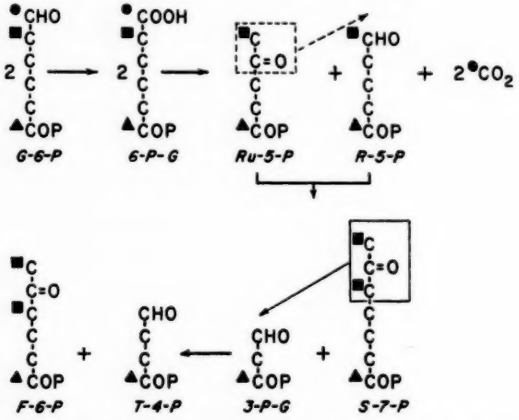


FIG. 1. Interrelations of carbon skeletons participating in the phosphogluconate oxidation pathway. G-6-P = glucose-6-phosphate, 6-P-G = 6-phosphogluconate, Ru-5-P = ribulose-5-phosphate, R-5-P = ribose-5-phosphate, S-7-P = sedoheptulose-7-phosphate, 3-P-G = 3-phosphoglyceraldehyde, T-4-P = tetrose-4-phosphate, F-6-P = fructose-6-phosphate. (Presumptive evidence has been presented that the expected aldotetrose, T-4-P, is D-erythrose-4-phosphate (18).)

cose after incubation with gluconate-6-C^{14} might result from a randomization incident to reversal of the Embden-Meyerhof sequence, commencing with triose-3-phosphate-3-C^{14} (Fig. 1).

In Table II are presented results of two experiments conducted with liver slices derived from a single rat. It was the intent of this study to compare the incorporation of C^{14} initially present in position 2 of glucose and in position 1 of ribose into carbons 1 and 6 of glycogen-glucose. As in the earlier experiment, carbon 2 of glucose contributed to a far greater extent to position 1 than to position 6. Similarly, carbon 1 of ribose was recovered in far higher yields in position 1 than in position 6 of glucose. These findings are again in complete accord with expectations based upon the phosphogluconate oxidation pathway (Fig. 1), in which carbon 1 of

ribose, assuming a preliminary phosphorylation at position 5, is derived from carbon 2 of 6-phosphogluconate or glucose-6-phosphate.

It has been suggested (19) that ribose-5-phosphate is degraded by rat liver acetone powder over the steps of the phosphogluconate oxidation pathway. The present results are taken to indicate, with a high degree of probability, that this same pathway is operating in the intact cells of the rat liver slice. The available evidence supports the contention that the alternative pathway, previously shown to account for about half of the glucose catabolized by liver slices (3), is the phosphogluconate oxidation pathway.

TABLE II

Incorporation of Isotope into Glycogen and CO_2

Incubation period, 3 hours. Other conditions as described in Table I. Values for single experiments. See Table I for foot-notes.

Substrate	Per cent radiochemical yield as CO_2	Specific activity* of glycogen carbons			
		1–6 (*a*)	1 (*b*)	6 (*c*)	2–5† (*d*)
Glucose-2-C^{14}	3.18	1760	935	39	2397
Ribose-1-C^{14}	3.12	505	1310	31	422

SUMMARY

Rat liver slices have been incubated with glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, gluconate-6-C^{14}, and ribose-1-C^{14}. The radioactivity of CO_2, fatty acids, and glycogen has been determined, and glycogen samples have been degraded to permit separate measurement of isotope in positions 1 and 6 of glucose.

Glucose-1-C^{14} yielded glycogen labeled predominantly in position 1, whereas glucose-6-C^{14} gave glycogen containing most of its isotope in position 6. Glucose-2-C^{14}, as well as ribose-1-C^{14}, yielded glycogen far more heavily labeled in position 1 of glucose than in position 6.

Gluconate-1-C^{14} failed to introduce detectable amounts of isotope into glycogen, whereas gluconate-6-C^{14} gave rise to glycogen labeled predominantly in position 6.

All of these findings are in accord with predictions based on the occurrence of the phosphogluconate oxidation pathway of glucose utilization in liver slices.

BIBLIOGRAPHY

1. Bloom, B., Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **204**, 681 (1953).
2. Bloom, B., and Stetten, D., Jr., *J. Am. Chem. Soc.*, **75**, 5446 (1953).

XUM

3. Bloom, B., and Stetten, D., Jr., *J. Biol. Chem.*, **212**, 555 (1955).
4. Eisenberg, F., Jr., *J. Am. Chem. Soc.*, **76**, 5152 (1954).
5. Horecker, B. L., *Brewers Digest*, **28**, 214 (1953).
6. Racker, E., *Advances in Enzymol.*, **15**, 141 (1954).
7. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **187**, 241 (1950).
8. Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, **154**, 669 (1944).
9. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, **194**, 69 (1952).
10. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).
11. Cook, M., and Lorber, V., *J. Biol. Chem.*, **199**, 1 (1952).
12. Gilvarg, C., *J. Biol. Chem.*, **199**, 57 (1952).
13. Dimant, E., Smith, V. R., and Lardy, H. A., *J. Biol. Chem.*, **201**, 85 (1953).
14. Topper, Y. J., and Hastings, A. B., *J. Biol. Chem.*, **179**, 1255 (1949).
15. Lorber, V., Lifson, N., Wood, H. G., Sakami, W., and Shreeve, W. W., *J. Biol. Chem.*, **183**, 517 (1950).
16. Stetten, M. R., and Topper, Y. J., *J. Biol. Chem.*, **203**, 653 (1953).
17. Racker, E., de la Haba, G., and Leder, I. G., *Arch. Biochem. and Biophys.*, **48**, 238 (1954).
18. Horecker, B. L., Smyrniotis, P. Z., Hiatt, H. H., and Marks, P. A., *J. Biol. Chem.*, **212**, 827 (1955).
19. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207**, 393 (1954).

FRACTION OF GLUCOSE CATABOLIZED VIA THE EMBDEN-MEYERHOF PATHWAY: ALLOXAN-DIABETIC AND FASTED RATS

By BEN BLOOM

(From the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York, New York, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland)

(Received for publication, November 18, 1954)

From the radiochemical yields of CO_2 and of fatty acids obtained when liver slices of normal rats are incubated with various specifically labeled precursors, it has been estimated (1) that about 50 per cent of the glucose molecules catabolized enter the Embden-Meyerhof sequence and the remainder follow an alternative pathway which is very similar or identical with the phosphogluconate oxidation pathway (2–4). The fraction of glucose molecules catabolized via the Embden-Meyerhof pathway (F or F') has been calculated by two independent equations. In the evaluation of F (Equation 2 (1)) the assumption was made that the alternative pathway was the phosphogluconate oxidation pathway. The evaluation of F' (Equation 4 (1)) required no such assumption.

Striking alterations in over-all carbohydrate metabolism are known to occur in diabetes and in fasting (5, 6). It was therefore considered of interest to carry out experiments which would permit estimation of F and F' for liver slices from diabetic and from fasted rats. To this end, the radiochemical yields of CO_2 and fatty acids from glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, gluconate-6-C^{14}, lactate-1-C^{14}, and lactate-3-C^{14} have been determined. From these quantities the rôle of the Embden-Meyerhof pathway in the over-all catabolism of glucose has been estimated.

EXPERIMENTAL

Treatment of Animals—The animals used in this study were adult male rats of the Sherman strain maintained on Purina chow. Diabetes was induced by the intravenous injection of a 5 per cent solution of alloxan monohydrate (50 mg. per kilo) to rats fasted overnight. During the 3 days prior to sacrifice, while the diabetic animals had access to a diet containing 60 per cent of glucose (7), measurements of urinary volume and total reducing value (8), expressed as gm. of glucose, were made (Table I). As will be seen from Table I, all the diabetic animals used in this study had

been injected with alloxan at least 1 month prior to sacrifice and had a very marked polyuria and glycosuria.

The fasted rats were stock animals previously maintained on Purina chow. Food was withheld from these animals for the periods indicated in Table II.

Procedures—The methods of preparation or sources of the substrates specifically labeled with C^{14} have been described (1, 7). Details of the incubation technique, CO_2 collection, fatty acid isolation, and of the assay methods for C^{14} have been reported (1).

TABLE I

History of Alloxan-Diabetic Rats

3 days prior to sacrifice the rats were offered a 60 per cent glucose diet. Urine volumes and glucose contents were determined.

Rat No.	Weight	Duration of diabetes	Urine					
			1st day		2nd day		3rd day	
			Volume	Glucose	Volume	Glucose	Volume	Glucose
	gm.	*days*	*ml.*	*gm.*	*ml.*	*gm.*	*ml.*	*gm.*
D1	170	30	190	19.4	260	39.0	230	34.5
D2	125	30	90	12.2	110	15.7	140	17.8
D3	150	30	205	30.7	260	38.0	255	36.6
D4	180	42	230	17.5	255	19.5	235	16.4
D5	175	42	170	13.0	230	16.7	200	17.9
D6	195	42	135	19.2	240	21.3	80	29.5
D7	170	58	205	27.9	280	38.0	230	27.0
D8	160	58	135	10.2	160	12.6	120	10.9

RESULTS AND DISCUSSION

The formation of CO_2 and fatty acids from specific carbons of glucose, gluconate, and lactate upon incubation with liver slices obtained from diabetic and from fasted rats is recorded in Table II. The mean values obtained with liver slices of normal rats have also been included. In comparison with the liver slices of normal rats, the livers of diabetic and fasted animals show a depression in the formation of $C^{14}O_2$ and fatty acid-C^{14} from glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14}. The inhibition of fatty acid synthesis is more marked than that of CO_2 formation. These findings are in accord with those reported for the utilization of glucose-U-C^{14} (9, 10).

The observations of Felts *et al.* (11) on the conversion of individual carbons of lactate to CO_2 and fatty acids by livers of diabetic rats are, for the most part, confirmed. It would appear in addition that an augmenta-

TABLE II

Conversion of Glucose-C^{14}, Gluconate-C^{14}, and Lactate-C^{14} to CO_2 and Fatty Acids by Liver Slices

Liver slices obtained from either diabetic or fasted rats were incubated with 5.0 ml. of bicarbonate buffer containing 50 μmoles of the radioactive substrate indicated below. The flasks containing isotopic glucose also contained 50 μmoles of non-isotopic lactate. The remaining flasks contained 50 μmoles of non-isotopic glucose. Radiochemical yields are calculated per 500 mg. of tissue. Each flask actually contained from 494 to 510 mg. of tissue. The incubation period for the experiments with labeled lactate was 2 hours; all other flasks were incubated 3 hours. The results are expressed in per cent.

Rat No.	Radiochemical yield from glucose						Radiochemical yield from gluconate			Radiochemical yield from lactate		
	-1-C^{14}		-2-C^{14}		-6-C^{14}		-1-C^{14}	-6-C^{14}		-1-C^{14}	-3-C^{14}	
	CO_2 (G_1)	Fatty acids ($\bar{G}_1$)	CO_2	Fatty acids	CO_2 (G_6)	Fatty acids	CO_2 (GT_1)	CO_2 (GT_6)	Fatty acids	CO_2 (L_1)	CO_2 (L_3)	Fatty acids ($\bar{L}_3$)
Diabetic rats												
D1	2.21	0.176	1.60	0.165	1.54	0.176	5.98	0.36	0.010			
D2	1.38	0.068	1.23	0.064	0.81	0.067	7.15	0.43	0.018			
D3	1.64	0.046	0.90	0.039	0.89	0.042	7.28	0.45	0.016			
D4	2.41	0.190			1.10	0.210				44.0	12.7	3.37
D5	0.73	0.021			0.32	0.028				40.7	13.8	1.68
D6	1.95	0.063			1.21	0.090				47.6	15.3	2.02
D7	1.60	0.070			0.41	0.068				26.0	7.10	1.75
D8	2.91	0.231			1.13	0.219				22.5	6.23	2.45
Fasted rats*												
F1	2.12				0.78					42.0	11.3	
F2	1.95				1.03					40.1	14.2	
F3	5.95	0.037			2.29	0.049				35.4	13.4	0.142
F4	4.61	0.023			1.65	0.030	8.65	1.03	0	33.8	12.9	0.148
F5	4.70	0.036			2.69	0.035	7.48	1.05	0	37.7	12.9	0.186
Normal rats†												
	18.0	3.6	9.72	5.3	4.10	4.8	5.89	0.73		28.4	6.22	10.0

In the experiments with gluconate-1-C^{14} and lactate-1-C^{14} no detectable isotope was present in the fatty acids.

* Rats F1, F2, and F3 were fasted 41 hours; Rats F4 and F5 for 24 hours.

† Average values obtained for normal rats (Tables I and III (1)).

tion in the formation of $C^{14}O_2$ from lactate-3-C^{14}, compared with normal values, has occurred not only in diabetic, but also in fasted animals. The decarboxylation of lactic acid, as evidenced by the normal or slightly enhanced production of $C^{14}O_2$ from lactate-1-C^{14}, taken together with a depressed lipogenesis from carbon 3 of lactate provides means for exaggeration of the other fates of carbon 3. These would include not only exalted ketogenesis, characteristically present in both diabetes and fasting, but also, as evidenced by the present data, oxidation to CO_2.

Fasting or the presence of diabetes did not reduce the conversion of gluconate-1-C^{14} or gluconate-6-C^{14} to $C^{14}O_2$ by liver slices. The formation of fatty acid from carbon 1 of gluconate could not be detected; that from carbon 6 would appear to be depressed if compared with yields procured earlier under slightly different experimental conditions (1).

In a study by Stetten and Topper (12) it was shown that in the normal intact rat the yield of $C^{14}O_2$ from gluconate-1-C^{14} was far greater than that from gluconate-U-C^{14}. The results of their studies with diabetic rats suggested, in contrast, that uniformly labeled gluconate formed $C^{14}O_2$ in greater yield than did gluconate-1-C^{14}. These latter findings, however, were not consistently reproducible. In the present studies involving CO_2 formation from carbons 1 and 6 of gluconate in liver slices from diabetic rats, no difference was noticed in the relative yields of $C^{14}O_2$ from gluconate-1-C^{14} and gluconate-6-C^{14} compared with normal values.

Fraction of Glucose Catabolized via Embden-Meyerhof Pathway—Two expressions for estimation of the fraction of catabolized glucose molecules which enter the Embden-Meyerhof pathway have been presented (1). The first expression, solving for F (Equation 2 (1)), made use of the assumption that the non-Embden-Meyerhof pathway is identical with the phosphogluconate oxidation pathway. The second expression, solving for F' (Equation 4 (1)), did not require this assumption. Determination of the following quantities was required for solution of these expressions: the radiochemical yields of CO_2 from glucose-1-C^{14} (G_1), glucose-6-C^{14} (G_6), gluconate-1-C^{14} (GT_1), gluconate-6-C^{14} (GT_6), lactate-1-C^{14} (L_1), and lactate-3-C^{14} (L_3) and the radiochemical yields of fatty acids from glucose-1-C^{14} ($\bar{G}_1$) and lactate-3-C^{14} ($\bar{L}_3$). It was shown (1) that if F or F' = the fraction of glucose catabolized via the Embden-Meyerhof pathway and if $G_6/G_1 = M$, $L_1/L_3 = N$, $GT_6/GT_1 = Q$, $\bar{G}_1/G_1 = H$, $L_1/\bar{L}_3 = I$, and $L_3/\bar{L}_3 = S$, then,

$$F = \frac{N(M - Q)}{N(M - Q) + (1 - M)}; \qquad F' = \frac{HI}{HI + 1 - HS}$$

The ratios required for solution of F and F' have been calculated from the radiochemical yields and are presented in Table III. The variables,

M, N, Q, H, I, and S, differ more or less in the diabetic and fasted animals from the mean values obtained with normal rats. In this regard, the most striking differences occur in those ratios involving $\bar{G}_1$ and $\bar{L}_3$, *i.e.*, H, I, and S. For example, the mean value of I changes from 5 in normals, to 17 in diabetics, to 210 in fasted liver slices. Despite this wide range in the values of the determinants of F', it will be noted that the values of F'

TABLE III

Fraction of Glucose, F or F', Catabolized via Embden-Meyerhof Pathway

The mean values, figures in parentheses, were used as indicated for calculation of F or F', according to Equations 2 and 4, respectively, of Bloom and Stetten (1).

Rat No.	M (G_6/G_1)	N (L_1/L_3)	Q (GT_6/GT_1)	H ($\bar{G}_1/G_1$)	I ($L_1/\bar{L}_3$)	S ($L_3/\bar{L}_3$)	F	F'
D1	0.698	(3.36)	0.060	0.0796	(17.0)	(5.23)	0.87	0.70
D2	0.587	(3.36)	0.060	0.0493	(17.0)	(5.23)	0.81	0.53
D3	0.542	(3.36)	0.062	0.0280	(17.0)	(5.23)	0.78	0.36
D4	0.457	3.46	(0.061)	0.0788	13.1	3.77	0.72	0.60
D5	0.438	2.95	(0.061)	0.0288	24.2	8.20	0.65	0.48
D6	0.621	3.11	(0.061)	0.0324	23.6	7.57	0.82	0.50
D7	0.256	3.66	(0.061)	0.0437	14.8	4.05	0.49	0.44
D8	0.388	3.61	(0.061)	0.0790	9.18	2.54	0.66	0.48
Mean							0.73	0.51
F1	0.368	3.72	(0.130)	(0.0063)	135	37.9	0.57	0.53
F2	0.528	2.82	(0.130)	(0.0063)	232	82.1	0.70	0.76
F3	0.385	2.64	(0.130)	0.0062	249	94.4	0.52	0.79
F4	0.358	2.62	0.119	0.0050	228	87.2	0.49	0.67
F5	0.572	2.92	0.142	0.0077	203	69.3	0.75	0.77
Mean							0.61	0.70
*	0.278	3.99	0.12	0.15	5.23	1.37	0.44	0.44

* Mean values obtained for normal rats (Table IV (1)).

are in reasonably good agreement with those of F. This finding is added support for the validity of the assumptions required in these derivations. Further supporting evidence is also available from studies involving the distribution of C^{14} in glycogen after incubation of liver slices with glucose-2-C^{14} and ribose-1-C^{14} (13).

The failure to obtain better agreement between F and F', 0.73 and 0.51, respectively, in the livers of diabetic rats makes it difficult to determine the extent of alteration from normal. Some suggestion of an increase in the fraction of glucose which is catabolized via the Embden-Meyerhof

pathway is apparent. With fasted rats mean values of 0.61 and 0.70 were obtained for F and F', respectively. These values, in good agreement, indicate that about 65 per cent of the glucose catabolized by fasted livers enters the Embden-Meyerhof pathway.

It is not possible from the present studies to determine whether the observed augmentation in the rôle of the Embden-Meyerhof pathway resultant to diabetes and fasting is *per se* a consequence of these pathological conditions. What rôle simple reduction in the quantity of glucose metabolized plays in determining the relative amounts catabolized via the Embden-Meyerhof and non-Embden-Meyerhof pathways is not apparent from the present study.

SUMMARY

1. Liver slices obtained from diabetic and from fasted rats have been incubated with the following substrates: glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, gluconate-6-C^{14}, lactate-1-C^{14}, and lactate-3-C^{14}.

2. The formation of CO_2 from glucose carbons 1, 2, and 6 was inhibited in diabetes or by fasting, while that from gluconate carbons 1 and 6 and lactate carbons 1 and 3 was either normal or augmented. Fatty acid synthesis was strikingly reduced from the substrates investigated.

3. By use of expressions previously presented it has been estimated that the fraction of catabolized glucose molecules which enter the Embden-Meyerhof pathway is increased in diabetes and in fasting.

BIBLIOGRAPHY

1. Bloom, B., and Stetten, D., Jr., *J. Biol. Chem.*, **212,** 555 (1955).
2. Horecker, B. L., *Brewers Digest*, **28,** 214 (1953).
3. Dickens, F., *Brit. Med. Bull.*, **9,** 105 (1953).
4. Racker, E., *Advances in Enzymol.*, **15,** 141 (1954).
5. Chaikoff, I. L., *Harvey Lectures*, **47,** 99 (1951–52).
6. Stadie, W. C., *Physiol. Rev.*, **34,** 52 (1954).
7. Bloom, B., Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **204,** 681 (1953).
8. Schales, O., and Schales, S. S., *Arch. Biochem.*, **8,** 285 (1945).
9. Chernick, S. S., Chaikoff, I. L., Masoro, E. J., and Isaeff, E., *J. Biol. Chem.*, **186,** 527 (1950).
10. Masoro, E. J., Chaikoff, I. L., Chernick, S. S., and Felts, J. M., *J. Biol. Chem.*, **185,** 845 (1950).
11. Felts, J. M., Chaikoff, I. L., and Osborn, M. J., *J. Biol. Chem.*, **191,** 683 (1951).
12. Stetten, M. R., and Topper, Y. J., *J. Biol. Chem.*, **203,** 653 (1953).
13. Bloom, B., Eisenberg, F., Jr., and Stetten, D., Jr., *J. Biol. Chem.*, **215,** 461 (1955).

XUM

METABOLISM OF I^{131} BY THE MARINE ALGA, NEREOCYSTIS LUETKEANA*

By W. TONG and I. L. CHAIKOFF

(From the Department of Physiology of the University of California School of Medicine, Berkeley, California)

(Received for publication, November 2, 1954)

Since the discovery of the element iodine in kelp by Courtois in 1811 (1), many reports dealing with the iodine composition of various marine plants have appeared (2–10). Interestingly enough, both the element itself and the first organic iodine compound known to occur naturally, diiodotyrosine, were observed in marine species before their presence was recognized in the thyroid gland (11). There is general agreement that iodine from the sea is concentrated in the tissues of these plants and that they contain iodine in both organic and inorganic forms. Recently, Roche and his coworkers demonstrated, with the aid of radioactive iodine and filter paper chromatography, that monoiodotyrosine and diiodotyrosine are formed by the marine alga, *Laminaria flexicaulis* (9).

The present report deals with the mechanisms involved in the concentration of iodide and the formation of organically bound iodine by the tissues of the marine alga, *Nereocystis luetkeana*.

EXPERIMENTAL

Specimens of *Nereocystis* were collected between November and June at Moss Beach approximately 20 miles south of San Francisco. Only the leaf-like fronds were used, and their most succulent and turgid portions were selected for study.

Treatment of Tissue

Intact squares of tissue and three types of tissue preparations were used. The latter are referred to as the whole homogenate, the washed homogenate, and the cell-free tissue extract.

Intact Tissue Squares—These were simply pieces approximately 1.5 cm. square by 1 mm. thick, cut from the plant fronds with scissors. Each square weighed approximately 300 mg.

Whole Homogenate—The whole homogenate was prepared by grinding 300 mg. squares of the tissue with 3 ml. portions of Krebs-Ringer-bicarbonate buffer. The grinding operations were performed in a Potter-Elvehjem type glass homogenizer. The resulting homogenate was imme-

* Aided by a grant from the United States Public Health Service.

diately transferred to a 25 ml. Erlenmeyer flask containing the I^{131}. The flask was then gassed with a 95 per cent O_2-5 per cent CO_2 mixture and placed in the incubation bath.

Washed Homogenate—When the whole homogenate was centrifuged at 3000 r.p.m. for 10 minutes, the iodine-metabolizing activity of the preparation was found to have sedimented with the insoluble material. In some experiments this insoluble material was resuspended in fresh bicarbonate-Ringer's buffer and incubated with I^{131}. This preparation is here termed the washed homogenate.

Cell-Free Extract—Although the simple Ringer's extract of the *Nereocystis* tissue was inactive in forming organically bound iodine, a highly active, cell-free preparation was obtained by subjecting the tissue to a more drastic treatment involving repeated grinding, freezing, and thawing. The details of this procedure are as follows: 200 gm. of tissue were homogenized in a Waring blendor with 1000 ml. of distilled water. The resulting slurry was centrifuged at 2000 r.p.m. for 15 minutes. The very viscous, pale green supernatant layer was discarded, and the residue was homogenized again with 300 ml. of water containing 200 mg. of $NaHCO_3$. This suspension was then frozen and stored overnight at $-17°$. The next day the material was allowed to thaw at room temperature. It was then thoroughly ground, 20 ml. at a time, in a homogenizing tube. This tube was made with thick glass walls and fitted with a Teflon pestle which was rotated at approximately 1500 r.p.m. The resulting homogenate was again frozen. The thawing, grinding, and freezing procedure was repeated the next day. Finally, on the 4th day, the material was thawed, transferred to 50 ml. centrifuge tubes, and centrifuged at 4500 r.p.m. for 20 minutes. Approximately 200 ml. of a clear, viscous, pale green extract were obtained. The extract was highly active in converting added inorganic I^{131} to the organic form, and it suffered no loss of activity after 4 months storage at $-17°$. Immediately before use, the frozen material was thawed at room temperature, and 3 ml. portions were incubated, with I^{131} and various substrates or inhibitors, in a gas phase of 95 per cent O_2 and 5 per cent CO_2.

Incubation Procedures

Each sample for incubation consisted of either a 300 mg. square of tissue immersed in 3 ml. of the Krebs-Ringer medium, or 3 ml. of one of the tissue preparations. The incubation mixtures were placed in 25 ml. Erlenmeyer flasks, and the radioiodine was added. Each flask was then flushed with the 95 per cent O_2-5 per cent CO_2 gas mixture, was stoppered, and agitated for 4 hours in a water bath at a temperature of 37°.

Radioactive iodide was added to the incubation mixtures as 50 or 100

μl. of a solution containing 20 to 150 μc. of carrier-free I^{131}. The larger amounts of radioactivity were used only when chromatographic analyses were carried out. Inhibiting agents, such as thiouracil, KSCN, etc., were added as 50 μl. of a 0.06 M solution made up in 0.154 M $NaHCO_3$. The tyrosine, monoiodotyrosine, thyronine, and diiodothyronine used in the tissue extract studies were added as 50 μl. of 0.1 M solutions made up in dilute NaOH.

Analytical Procedures

Determination of Radioiodide-Concentrating Capacity—The I^{131} concentration ratios were determined in the experiments with tissue squares. At the end of the incubation period, the medium from each sample was decanted into a volumetric flask, and the tissue squares were quickly washed with two 10 ml. portions of fresh Ringer's buffer. The washings were combined with the medium for I^{131} assay. Each square was then homogenized with 3 ml. of ice-cold, 20 per cent trichloroacetic acid (TCA). After centrifugation at 4000 r.p.m. for 5 minutes, the supernatant phase, which was found by means of paper chromatography to contain only inorganic I^{131}, was decanted into a volumetric flask. The precipitate was washed once with 5 ml. of 10 per cent TCA and then dissolved in 2 N NaOH for I^{131} assay. Radioactivity assays were performed, with a scintillation type detector, directly on solutions of the I^{131}-containing samples delivered into vials. The I^{131} content of the combined TCA supernatant fraction and washings represented the inorganic I^{131} of the tissue, while the I^{131} in the TCA precipitate constituted the organic I^{131} formed by the tissue. The I^{131} remaining in the buffer medium at the end of the incubation was also found by means of chromatography to be entirely inorganic in nature. Hence, the I^{131}-concentrating capacity of the tissue square is given by the ratio,

$$\frac{\text{TCA-soluble } I^{131} \text{ per gm. tissue}}{I^{131} \text{ per ml. medium}}$$

Determination of Organically Bound I^{131}—The above procedure also served for the determination of the organic I^{131} fraction in the experiments carried out with the intact tissue squares, whole homogenates, and the washed homogenates. This procedure could not, however, be applied to the cell-free tissue extracts because the content of TCA-precipitable material in those extracts was found to be quite low.

Chromatographic Procedures—Further information on the chemical nature of the organic I^{131} formed during incubation was obtained by proteolytic hydrolysis and paper chromatography. At the end of the incubation, 100 μl. of 0.06 M thiouracil were added to each mixture to prevent further

reaction. The samples were then diluted with 0.154 M $NaHCO_3$ to yield a final concentration of 50 mg. of tissue per ml. The intact tissue square samples were homogenized in a glass homogenizing tube at this point. The pH of all samples was adjusted to 8 to 8.5. 0.5 ml. aliquots of the resulting suspensions were digested with 10 mg. of pancreatin (Merck) for 10 to 15 hours at 37°. Toluene was added to prevent putrefaction. At the end of the digestion period, 20 μl. portions of the hydrolysates were delivered onto strips of Whatman No. 1 filter paper and developed with a collidine and water mixture. Details of the chromatographic procedure have been reported previously (12). The I^{131} content of each component on the filter paper chromatogram was determined with the aid of a strip-counting device described elsewhere (13).

Results

Concentration of Radioiodide by Intact Tissue

Squares of *Nereocystis* tissue incubated in buffer containing radioiodide were capable of attaining internal *radioiodide* concentrations 6 to 10 times those of the surrounding buffer media (Table I). The mechanism responsible for concentrating inorganic iodide was inhibited by 0.001 M thiouracil, sulfite, cyanide, thiocyanate, and sulfathiazole, and by the exclusion of oxygen. It was not inhibited by 0.001 M azide, sulfanilamide, sulfaguanidine, or perchlorate.

Formation of Organic I^{131} by Intact Tissue and by Homogenates

After 4 hours of incubation in the I^{131}-labeled bicarbonate-Ringer's solution, intact *Nereocystis* tissue contained 42.1 to 51.4 per cent of the added tracer I^{131} in TCA-insoluble form (fifth column, Table I). The formation of TCA-insoluble I^{131} (presumably organic) was inhibited in the presence of 0.001 M thiouracil, sulfite, cyanide, thiocyanate, sulfathiazole, and by anaerobic conditions. The same concentration of azide, sulfanilamide, sulfaguanidine, and perchlorate had no effects. Since both uptake and organic binding of radioiodide were inhibited by the same compounds, it is possible that the inhibition of either process resulted from an inhibition of the other.

Similar results were observed in the experiments with tissue homogenates, although the actual values for organic I^{131} formation were more variable.

Chromatographic analysis of the various incubation mixtures before hydrolysis showed the presence of only two distinct components: inorganic I^{131} at $R_F = 0.8$, and organic I^{131} at the origin. The fact that the organic I^{131} was not moved by the chromatographing solvent indicated that it was

probably protein in nature, and for this reason the incubation mixtures were subjected to hydrolysis with pancreatin. Radioautographs of chromatograms prepared from the enzyme digests are shown in Fig. 1. The

TABLE I

Metabolism of Tracer Radioiodide by Intact Nereocystis Tissue

Experiment No.	Conditions	Per cent added I^{131} taken up by tissue	Radioiodide concentration ratio, TCA-soluble I^{131} per gm. tissue / I^{131} per ml. media	Organic iodine formed*
				per cent added I^{131}
3	Control	68.8	8.5	42.1
	"	71.5	9.4	44.6
	0.001 M thiouracil	7.5	0.7	1.1
	0.001 " "	7.5	0.8	0.5
	0.001 " $KClO_4$	68.6	8.4	42.8
	0.001 " "	72.4	7.9	49.4
	0.001 " KSCN	10.2	0.8	2.8
	0.001 " "	9.8	0.9	1.9
10	Control	72.0	9.8	44.3
	"	73.4	10.0	47.8
	"	74.8	9.5	51.4
	"	70.7	8.6	45.7
	0.001 M Na_2SO_3	12.6	0.9	4.9
	0.001 " "	10.7	0.8	3.3
	0.001 " "	11.5	0.9	3.9
	0.001 " NaCN	19.1	1.3	8.7
	0.001 " "	20.2	1.4	9.4
	0.001 " "	15.7	1.1	7.2
	0.001 " NaN_3	75.4	9.6	52.1
	0.001 " "	73.5	8.6	50.8
	0.001 " "	73.2	8.0	51.7
	0.001 " sulfanilamide	72.5	7.7	51.5
	0.001 " sulfaguanidine	75.9	9.1	53.4
	0.001 " sulfathiazole	25.1	1.5	13.6
13	Control	65.4	6.1	44.4
	"	64.7	6.4	42.1
	"	65.2	6.0	44.6
	Anaerobic	17.6	1.2	7.3
	"	12.6	1.0	3.8

* TCA-insoluble fraction.

major products released by the hydrolytic treatment in each case have been identified as monoiodotyrosine and diiodotyrosine by the usual technique of cochromatography with added pure compounds. Fig. 1 shows that, regardless of whether intact tissue, homogenate, or tissue extract was

used, both iodotyrosines were formed. However, in proceeding from intact tissue to cell-free extract, the formation of the monoiodinated derivative became dominant. This observation is presented in a more quantitative form in Table II, in which the values were obtained by assaying the radioactivity in each component on the chromatograms with the strip-counting device.

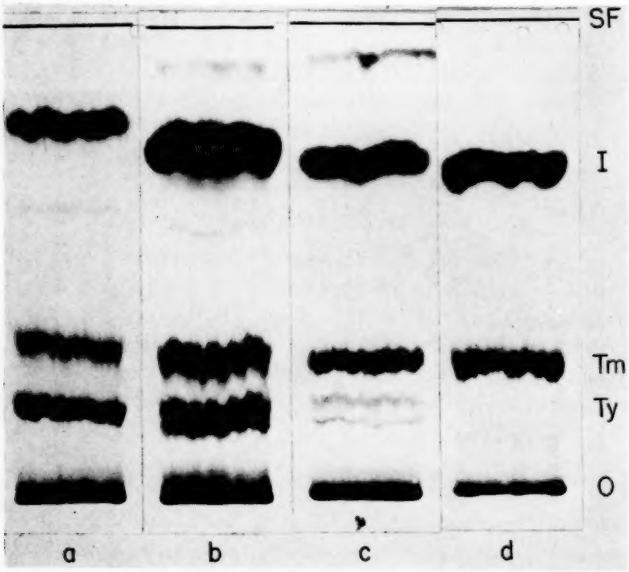


FIG. 1. Radioautograph of chromatograms of pancreatin hydrolysates of *a*, intact square; *b*, whole homogenate; *c*, washed homogenate; and *d*, cell-free extract of *Nereocystis* after incubation with carrier-free I^{131}. O, origin; Ty, diiodotyrosine; Tm, monoiodotyrosine; I, inorganic iodine; SF, solvent front. The faint components near the solvent front and below inorganic iodine have not been identified.

The I^{131}-containing material that remains at the origin of the chromatograms (Fig. 1) is very probably iodinated protein that had escaped hydrolysis by the enzymes. The faint component appearing immediately under the inorganic iodide bands at $R_F = 0.6$ has not been identified. Although its position on the chromatogram was close to that occupied by added pure thyroxine, its faintness rendered positive identification by the usual techniques extremely difficult. The component appearing just below the solvent front on the chromatograms of the homogenates (Fig. 1, *b* and *c*) has also not been identified.

Formation of Organic I^{131} by Cell-Free Extracts

Cell-free extracts converted about 20 per cent of the added I^{131} to organic form. These figures were obtained by chromatographic analysis because, as pointed out above, there was very little TCA-precipitable material in the enzyme extract. Chromatograms prepared from pancreatin hydrolysates of the incubation mixtures revealed that varying amounts of monoiodotyrosine and diiodotyrosine had been formed. A radioautograph of one such chromatogram is reproduced in Fig. 1, *d*. Some of the chromatograms were assayed for radioactivity with the strip counter, and the results are shown in Table II.

TABLE II

Composition of Pancreatin Hydrolysates of I^{131}-Labeled Nereocystis Preparations

Experiment No.	Tissue preparation	Per cent added I^{131} recovered as				
		Protein	Diiodotyrosine	Monoiodotyrosine	R_F 0.6	I^-
6	Intact squares	13.4	3.8	7.0	0.4	75.5
	Whole homogenate	10.5	1.6	3.7	0.6	83.8
	Washed "	27.5	7.5	20.0	1.1	40.0
13	Intact squares	15.8	5.8	6.2	0	71.5
	Whole homogenate	10.9	1.2	1.7	0	86.2
	Washed "	7.9	0.5	0.8	0	90.8
13	Cell-free extract	10.8	0.8	4.8	0	82.9
	" "	10.6	0.8	4.3	0	84.2

Iodination of Added Tyrosine, Monoiodotyrosine, and Thyronine by Cell-Free Extracts

The use of the cell-free extract enabled us to study the iodination of a number of compounds. The results are illustrated by the radioautographs reproduced in Fig. 2. Figs. 2, *a* and 2, *b* are controls, and they show that no free iodinated amino acids are formed by the boiled extract incubated with I^{131} and tyrosine, nor by the unboiled extract incubated with I^{131} alone. When, however, 0.0015 M tyrosine was added to the latter, monoiodotyrosine was formed (Fig. 2, *c*). An assay of the radioactivity on this chromatogram showed that 30 per cent of the added I^{131} was incorporated into the monoiodotyrosine component. Similar results were obtained with D- and L-tyrosine, indicating that the iodinating system is not specific with respect to optical isomerism.

Added L-monoiodotyrosine was converted to labeled diiodotyrosine (Fig. 2, *d*), but only to the extent of 15 per cent of the added I^{131}. The chro-

matogram of Fig. 2, *d* also shows little, if any, radioactivity appearing in the monoiodotyrosine region. This finding indicates that exchange type reactions do not occur to a significant degree under the conditions of these experiments.

Added L-diiodothyronine was very slightly iodinated; chromatograms of the reaction mixture showed faint bands in the triiodothyronine and thyroxine regions. This reaction was complicated by the fact that the added material could not be kept in solution. When L-thyronine was incubated

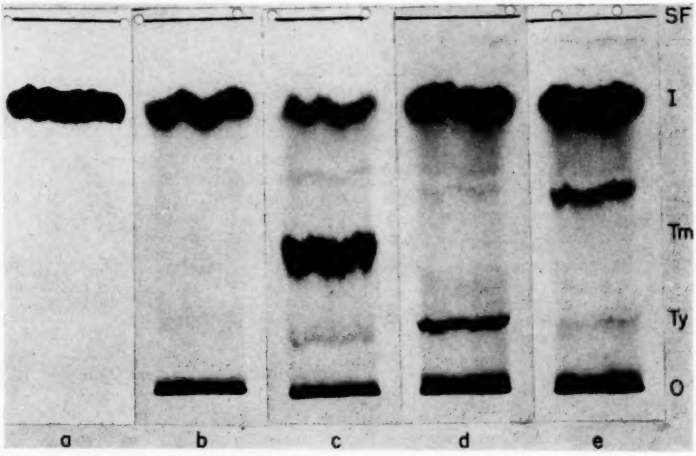


FIG. 2. Radioautographs of chromatograms of the cell-free extract of *Nereocystis* incubated with carrier-free I^{131} and various added substrates. *a*, boiled extract incubated with I^{131} and tyrosine; *b*, control extract incubated with I^{131} alone; *c*, extract incubated with I^{131} and 0.0015 M tyrosine; *d*, extract incubated with 0.0015 M monoiodotyrosine; *e*, extract incubated with 0.0015 M thyronine. Symbols as for Fig. 1.

with the cell-free extract, a new component appeared which accounted for about 10 per cent of the added I^{131} (Fig. 2, *e*). This component had approximately the same R_F on the chromatograms as does thyroxine. However, when pure thyroxine was chromatographed along with the sample, and when the resulting chromatogram was sprayed with a diazotized sulfanilic acid reagent, the thyroxine color band was slightly above the radioactive band of the new component. It seems likely that this component is another iodinated derivative of thyronine, perhaps a monoiodothyronine.

The iodinating activity of the cell free extract, upon its own protein matter as well as upon added tyrosine, was inhibited by 0.001 M concen-

trations of thiouracil, thiocyanate, cyanide, sulfathiazole, and by the exclusion of oxygen. Furthermore, it was completely inactivated by immersion for 3 minutes in a boiling water bath. Because it seemed possible that a peroxidase type of reaction was involved here, the effect of adding catalase to the cell-free preparation was investigated. Approximately 0.25 mg. of a crystalline catalase preparation (General Biochemicals, Inc.) completely inhibited the iodinating action of a 3 ml. portion of the cell-free extract. On the other hand, azide and the sulfonamide drugs, which inhibit horseradish peroxidase (14), had no effect on the *Nereocystis* enzyme. The inhibiting action of sulfathiazole seems to involve the formation of an iodinated sulfathiazole. Chromatograms of the cell-free preparation incubated with I^{131}, tyrosine, and sulfathiazole showed the formation of a new compound located above iodide. This component contained 45 per cent of the added I^{131}, and could be formed simply by incubating sulfathiazole with I^{131} in the presence of 0.001 M H_2O_2.

Experiments with Other Plants

It seemed of interest to determine the extent to which the ability to metabolize I^{131} existed in other plant tissues. A number of studies similar to those described above were performed with the fresh water, coenocytic alga, *Nitella*, the aquatic angiosperm, *Lemna*, and slices of horseradish root. Of these, only the *Nitella* was found to utilize I^{131}. The best results were obtained when this material was incubated in fresh pond water at room temperature.

After 4 hours of incubation in pond water containing carrier-free I^{131}, the *Nitella* tissue took up approximately 50 per cent of the added I^{131}. Of the I^{131} taken up, 60 per cent was present in TCA-insoluble fraction, which was found, after hydrolysis with 2 N NaOH, to contain monoiodotyrosine but no diiodotyrosine. During the 4 hour incubation period, this plant attained I^{131} concentration ratios that varied between 3 and 6 as calculated by the method described above. Both the concentration and organic binding of I^{131} by this material were inhibited by 0.001 M concentrations of thiouracil, thiocyanate, and cyanide.

DISCUSSION

The results of the present investigation indicate that the mechanism responsible for iodide concentration in *Nereocystis* is quite different from that operating in the thyroid gland. This was shown by the finding that the iodide-concentrating capacity in the plant was depressed by thiouracil but unaffected by perchlorate, while the opposite response to these agents is obtained with the thyroid (15). However, as in the case of the thyroid, the inorganic iodine concentrated in the plant tissue appears to exist there

in a form indistinguishable, by our techniques, from iodide. Some form of active ion transport seems to be involved which is dependent upon respiratory function, since the trapping mechanism is completely blocked by anaerobic conditions.

Approximately one-third of the radioiodide taken up by intact squares of *Nereocystis* frond during 4 hours of incubation was found in the inorganic form, and two-thirds in an organically bound form. It is reasonable to assume that the I^{131} entered the tissue as iodide, and there became oxidized before being converted to an organic form. There are many reports dealing with the ability of various plant tissues to oxidize added iodide to iodine (3–7, 16, 17). In these earlier experiments, a starch-iodide reagent was applied either directly to tissues or to extracts of tissues, and the appearance of the characteristic blue starch-iodine color was noted. More recently, Roche, van-Thoai, and Lafon (8) titrated with thiosulfate the I_2 released by pieces of seaweed tissue incubated in solutions of KI. However, a clear-cut demonstration of the presence of I_2 within the plant tissues has not, as yet, been reported. In this laboratory, an attempt was made to extract radioactive I_2 from *Nereocystis* tissue incubated in bicarbonate-Ringer's buffer containing carrier-free radioiodide. None of the I^{131} taken up by the tissue after incubation periods ranging from 25 minutes to 4 hours could be extracted with carbon tetrachloride. This situation should be compared with that in the thyroid gland, where iodination certainly occurs, and yet, with our chromatographic and extraction techniques, oxidized forms of iodide cannot be detected.

Bach and Chodat identified the iodide-oxidizing agent in the plant tissues as an oxidase (16, 17), and suggested that the mechanism of oxidation involved first the formation of H_2O_2 by the oxidase, and then the oxidation of iodide by the H_2O_2. For the marine alga, *Laminaria*, Roche *et al.* proposed that cytochrome oxidase was involved because the iodide oxidation was inhibited by carbon monoxide, in the dark, and by homogenization of the tissue. Our findings with *Nereocystis*, however, are more in agreement with those of Bach and Chodat. The inhibition by crystalline catalase implicates H_2O_2, whereas cytochrome oxidase does not produce H_2O_2. Furthermore, cytochrome oxidase would have been inhibited by azide.

In an attempt to assay the rôle of H_2O_2 in iodination reactions, we studied a non-enzymatic iodinating system with low concentrations of H_2O_2. When 0.001 M L-tyrosine, dissolved in bicarbonate-Ringer solution, was incubated with 0.001 M H_2O_2 and carrier-free radioiodide, approximately 25 per cent of the added I^{131} was incorporated into monoiodotyrosine. When the H_2O_2 concentration was reduced to 10^{-4} M, no iodination occurred. These findings indicate that the organic binding of I^{131} by the

plant could proceed through a similar peroxide oxidation of iodide, provided that the tissue is able to attain a peroxide concentration of at least 10^{-3} M. It is possible that even lower concentrations of H_2O_2 would suffice if there were available peroxidase type enzymes capable of catalyzing the oxidation of iodide. This possibility must be considered since peroxidases have been shown to occur in high concentrations in marine algae (18). The effects of peroxidase inhibitors were investigated in the hope of clarifying this point. Although certain of the inhibitors (thiouracil, cyanide, and thiocyanate) were found to inhibit the iodine-metabolizing function of the plants, these same agents were equally effective against the non-enzymatic iodinating system. On the other hand, the fact that azide ion, another peroxidase inhibitor, had no apparent effect on the plants indicates that peroxidase action is not involved.

It is interesting that, with the non-enzymatic iodination system as well as with the *Nereocystis* extract and the various thyroid tissue preparations reported (19–21), the sole product obtained in the iodination of tyrosine is the monoiodinated derivative. It is also worthy of note that, with the exception of catalase, all of the chemicals found to inhibit iodination reactions in the non-enzymatic and the *Nereocystis* systems are also known inhibitors of thyroid function.

We are indebted to Dr. R. Pitt-Rivers for the 3-monoiodo-L-tyrosine used in our experiments, to Dr. E. L. Sevringhaus of Hoffmann-La Roche, Inc., for the L-thyronine, and to Dr. B. A. Hems of Glaxo Laboratories, Ltd., for 3,5-diiodo-L-thyronine. It is a pleasure to acknowledge the many helpful discussions with Dr. Alvin Taurog during the course of this investigation.

SUMMARY

1. Radioiodine and filter paper chromatography were applied to an investigation of iodine metabolism by the marine alga, *Nereocystis luetkeana*.

2. Portions of tissue from this plant, when incubated in Krebs-Ringer-bicarbonate buffer containing carrier-free I^{131}, attained radioiodide concentrations 6 to 10 times that of the incubating media.

3. In addition to the I^{131} present in inorganic form, the incubated tissues contained varying amounts of I^{131} in organic combination. In agreement with observations of other investigators, the organic I^{131} was shown to yield principally monoiodotyrosine and diiodotyrosine upon hydrolysis.

4. When the tissue was ground up, its capacity to bind iodine organically was found to be firmly associated with the insoluble residue. However, an active, cell-free extract was obtained by subjecting the tissue to

repeated grinding, freezing, and thawing. This extract was capable also of iodinating added tyrosine, monoiodotyrosine, and thyronine.

5. The concentration of iodide by the intact tissue squares and the formation of organic iodine by tissue squares, homogenates, and extract were inhibited by 0.001 M concentrations of thiouracil, sulfite, cyanide, thiocyanate, and sulfathiazole, and by the exclusion of oxygen. In addition, the iodinating action of the cell-free tissue extract was completely inhibited by crystalline catalase.

6. The probable involvement of H_2O_2 in the formation of organic I^{131} by *Nereocystis* is discussed.

BIBLIOGRAPHY

1. Courtois, M. B., *Ann. chim.*, **88,** 304 (1813).
2. Eschle, K., *Z. physiol. Chem.*, **23,** 30 (1897).
3. Kylin, H., *Z. physiol. Chem.*, **94,** 341 (1915).
4. Dangeard, P., *Bull. Soc. bot. France*, **75,** 509 (1928).
5. Mangenot, G., *Bull. Soc. bot. France*, **75,** 519 (1928).
6. Chemin, E., *Rev. gên. bot.*, **40,** 129 (1928).
7. Kylin, H., *Z. physiol. Chem.*, **186,** 50 (1930).
8. Roche, J., van-Thoai, N., and Lafon, M., *Compt. rend. Soc. biol.*, **143,** 1327 (1949).
9. Roche, J., and Yagi, Y., *Compt. rend. Soc. biol.*, **146,** 642 (1952).
10. Scott, R., *Nature*, **173,** 1098 (1954).
11. Drechsel, E., *Z. Biol.*, **33,** 85 (1896).
12. Taurog, A., Tong, W., and Chaikoff, I. L., *J. Biol. Chem.*, **184,** 83 (1950).
13. Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **207,** 59 (1954).
14. Lipmann, F., *J. Biol. Chem.*, **139,** 977 (1941).
15. Wyngaarden, J. B., Wright, B. M., and Ways, P., *Endocrinology*, **50,** 537 (1952).
16. Bach, A., and Chodat, R., *Ber. chem. Ges.*, **35,** 2466 (1902).
17. Chodat, R., and Bach, A., *Ber. chem. Ges.*, **35,** 3943 (1902).
18. Rönnerstrand, S., Untersuchungen über Oxydase, Peroxydase und Ascorbinsäure in einigen Meersalgen, Lund (1943).
19. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, **205,** 795 (1953).
20. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, **209,** 249 (1954).
21. Taurog, A., Potter, G. D., and Chaikoff, I. L., *J. Biol. Chem.*, **213,** 119 (1955).

STUDIES ON THE STRUCTURE OF THE PHYTOPATHOGENIC TOXIN OF PSEUDOMONAS TABACI

By D. W. WOOLLEY,* G. SCHAFFNER, and ARMIN C. BRAUN

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, New York)

(Received for publication, January 6, 1955)

A pathogenic factor in wildfire disease of tobacco, the toxin of *Pseudomonas tabaci*, which has been shown to be an antagonist of methionine (1), has recently been isolated in pure form (2). A major constituent of this toxin has been identified as the new amino acid tabtoxinine, or α, ϵ-diamino-β-hydroxypimelic acid (3). The object of the present study was to determine the entire structure of the toxin. The results indicated that the structure is that shown in Fig. 1.

The following kinds of evidence led to the proposal of this formula: (*a*) Elementary analysis of the toxin gave values which would correspond to $C_{10}H_{16}O_6N_2$. Because no satisfactory method for the determination of molecular weight could be applied, this empirical formula was the minimal one possible. There was some doubt about the reliability of the analytical figures because of the inauspicious properties of the isolated toxin (2).

(*b*) After hydrolysis, at least 55 per cent of the dry weight of the toxin could be isolated as tabtoxinine (3). Clearly, then, this amino acid represented a major part of the toxin.

(*c*) One-half of the total nitrogen of the toxin was α-amino nitrogen (2). This suggested that one of the amino groups of tabtoxinine was free and the other was combined. Acid hydrolysis rendered almost all of the nitrogen in a form determinable as α-amino nitrogen.

(*d*) The products of complete hydrolysis with acid were isolated and found to be tabtoxinine and lactic acid. Small amounts of formic acid were also identified, but these were considered to have arisen from decomposition of the major components. Diligent search failed to reveal any other constituent. For example, no evidence for a volatile alcohol was found in the methoxyl determination. Similarly, no other acidic components could be detected.

(*e*) Different hydrolytic procedures gave two different ninhydrin-positive substances. The one produced by strong acid was tabtoxinine. The other was formed when the toxin was inactivated by very weak alkali (2). This latter substance, called the alkali-inactivated toxin, was converted to tabtoxinine by either strong acid or strong alkali. It was thus a product of partial cleavage (see Fig. 1).

* With the technical assistance of E. Van Winkle.

(*f*) The toxin was a neutral compound, giving aqueous solutions of approximately pH 6 (2) and migrating much as leucine did in paper electrophoresis. On the other hand, the alkali-inactivated toxin was an acid.

(*g*) Although tabtoxinine was destroyed readily by periodate (3), both the toxin and the alkali-inactivated toxin were not. This (along with (*c*)) indicated that, in both of these substances, the α-amino group of tabtoxinine carried some substituent. Consequently, it must have been the ϵ-amino group which was the one found free in the analytical procedures.

(*h*) This point was corroborated in the following way. The 2,4-dinitrophenyl derivative (DNP toxin) was prepared. Dilute alkali converted this to a substance indistinguishable from the DNP derivative prepared

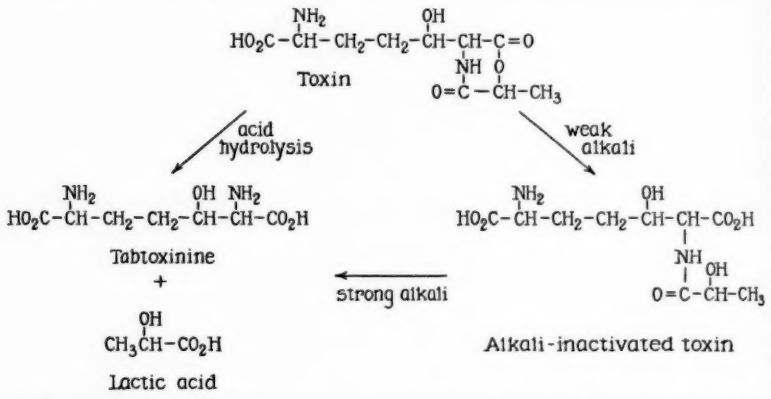


FIG. 1. Structure of the toxin of *P. tabaci* and some reactions leading to this formulation.

from alkali-inactivated toxin. Acid hydrolysis of this DNP-alkali-inactivated toxin yielded a DNP-amino acid which contained a free amino group. This substance, which was ϵ-DNP-tabtoxinine, was readily destroyed by periodate. This property distinguished it from α-DNP-tabtoxinine, and showed that in the toxin it was the ϵ-amino group of tabtoxinine which was free.

(*i*) Quantitative determination showed that, in both the toxin and the DNP-alkali-inactivated toxin, there was 1 lactic acid residue per molecule. This lactyl group, therefore, must have been attached to the α-amino group of tabtoxinine.

(*j*) The formulation of the toxin as a lactone was based on the ease of alkaline cleavage, on the failure to find any constituent other than lactic acid and tabtoxinine, and on the fact that the toxin was neutral whereas the alkali-inactivated toxin was acidic. Conceivably, several lactones

could account for these properties. The involvement at the α-carboxyl, and of necessity the hydroxyl of the lactyl residue, seemed most plausible and was supported by (*k*). Much effort to demonstrate the existence of a β-lactone in the toxin failed to reveal one.

(*k*) Either one of the carboxyl groups of the tabtoxinine could be the one involved in the lactone linkage. The assignment of this function to the α-carboxyl was based on the following evidence. The ninhydrin reaction of the toxin was abolished by prior treatment with copper carbonate in the manner described by Crumpler and Dent (4). If the ϵ-carboxyl group of tabtoxinine had been the one involved in the lactone linkage, then the toxin would not be an α-amino acid and should not react in the way just described.[1]

Although consideration of this evidence has led to the tentative structure proposed for the *P. tabaci* toxin, a few points remained obscure. For this reason, the structure was regarded as tentative rather than final. The DNP toxin was very soluble in water and was not extracted by non-polar solvents. On the other hand, the DNP-alkali-inactivated toxin was less soluble in water and extractable by ethyl acetate. One would not expect to find these differences.

The small amount of formic acid formed during hydrolysis of a toxin with acid probably arose from secondary decomposition. The quantity found never exceeded 0.4 mole per mole of toxin. In fact, under the standard conditions for hydrolysis in the formyl determination (5), the toxin was found to contain only 3.6 per cent formyl (0.32 mole). When the time of hydrolysis was increased 4-fold, the higher value was found. The DNP-alkali-inactivated toxin yielded only a trace of formic acid. This was interpreted to mean that substitution of the ϵ-amino group of tabtoxinine had rendered the molecule less likely to decompose with the production of formic acid.

The occurrence of lactic acid in hydrolysates of the toxin was missed for some time for the following reason. It was known that acid hydrolysates contained a non-volatile, ether-extractable acid, the R_F of which was the same as for lactic acid. However, the quantitative colorimetric determination of lactic acid in ether extracts of the hydrolysate failed to show any. This failure proved to be due to an interfering substance, because added lactic acid could not be recovered. Alkaline hydrolysis followed by ether extraction of lactic acid avoided this difficulty.

If the proposed structure for the toxin is admitted, then the original working hypothesis of this study has been verified. This hypothesis was that the toxin was a naturally occurring antimetabolite of methionine (2).

[1] The α-amino group of tabtoxinine has, in the toxin, been acylated, and hence is no longer free to react with ninhydrin.

The fact that it does bear a structural analogy to methionine (*cf.* also (3)) and that its harmful action on plants such as *Chlorella vulgaris* is competitively reversed by methionine would be the evidence for such a point of view.

EXPERIMENTAL

Toxin was isolated according to the directions of Woolley *et al.* (2), and was stored at −16°. It was necessary to carry out twenty-seven preparations in order to accumulate sufficient material. All paper chromatography was carried out by the ascending method unless otherwise stated. Whatman No. 1 paper was used. All reactions with periodate were carried out in aqueous solutions adjusted to pH 7. The unknown being tested was present at 1 mg. per cc., sodium periodate ($NaIO_4$) was added in 50 per cent excess, and the reaction mixtures were applied to filter papers after they had stood at room temperature for at least 30 minutes. All melting points were determined with a heated stage microscope.

Empirical Formula—As described earlier (2), elementary analysis of the toxin was difficult because of its hygroscopic nature and its instability. A further source of trouble was found in that the Amberlite resin used in the final purification contributed nitrogenous impurities. Thorough washing of this resin and reduction of the amount used to the minimum necessary for removal of sulfate ions led to toxin with a nitrogen content somewhat lower than previously described (2). The following values are believed to represent the true ones, although, because of the character of the toxin, they must be viewed with some doubt.[2]

$C_{10}H_{16}O_6N_2$.	Calculated.	C 46.2, H 6.15, N 10.8
	Found.	" 46.0, " 6.05, " 11.2

Alkali-Inactivated Toxin—100 mg. of toxin were dissolved in 10 cc. of 0.4 M $NaHCO_3$ and the solution was allowed to stand at 25° for 3 hours. If the pH fell at the start, brief evacuation of the flask served to restore alkalinity. An aliquot of the reaction mixture was diluted to 1 mg. of toxin per cc. and a spot (0.01 cc.) was developed alongside a spot of untreated toxin in an ascending chromatogram with Solvent A of Table I. When the developed chromatogram was sprayed with ninhydrin and heated at 100°, a purple spot of R_F 0.18 marked the position of the alkali-inactivated toxin. In the same system, the toxin had R_F 0.26 (2). During the development of the chromatogram, the main reaction mixture was stored at −16° to minimize further hydrolysis to tabtoxinine. If no spot at R_F 0.26 remained (toxin), and none at R_F 0.06 (tabtoxinine) had appeared, the solution was ready for further use. Little difficulty was encountered

[2] We are much indebted to Mr. T. Bella for all elementary analyses.

in the preparation of chromatographically pure alkali-inactivated toxin in this way. The toxic activity as measured on tobacco leaves (2) was entirely lost. Crystalline alkali-inactivated toxin could not be isolated.

DNP-Alkali-Inactivated Toxin—A solution prepared as described above was treated with 200 mg. of $NaHCO_3$ and a solution of 200 mg. of 2,4-dinitrofluorobenzene (DNFB) in 20 cc. of ethanol and allowed to stand at room temperature for 20 hours. The yield was improved by this longer than usual (*cf.* (6)) reaction time. The solvent was removed under reduced pressure (bath temperature, 37°), and the residue was suspended in 15 cc.

TABLE I

R_F's of Products Derived from Toxin

Compound	Solvent					
	A	B	C	D	E*	F
Alkali-inactivated toxin	0.18					
DNP-alkali-inactivated toxin		0.22	0.67	0.73	0.90	
DNP toxin				0.63	1.0	
ε-DNP-tabtoxinine	0.32†	0.0			0.12	0.75
di-DNP-tabtoxinine	0.64	0.17	0.70			

Solvent A, 200 cc. of *n*-propanol + 100 cc. of water; Solvent B, 100 cc. of *n*-butanol + 95 cc. of water + 5 cc. of concentrated aqueous ammonia; Solvent C, 100 cc. of *n*-butanol + 90 cc. of water + 10 cc. of acetic acid equilibrated 5 hours; Solvent D, 100 cc. of *n*-butanol + 100 cc. of 1 N HCl; Solvent E, equal volumes of *n*-butanol + formic acid + water, equilibrated 3 days (*cf.* (8)); Solvent F, equal volumes of ethanol + *n*-butanol + formic acid + water, equilibrated 3 days.

* Descending method.

† This was the R_F when the free amino acid was used; the hydrochloride gave 0.48.

of water and extracted three times with benzene, and then six times with ether. The aqueous phase was adjusted to pH 4 with HCl, and extracted twice with benzene. The benzene removed dinitrophenol. The resulting aqueous phase was adjusted to pH 2.0 with HCl, and the DNP-alkali-inactivated toxin was extracted with 5 portions of ethyl acetate. Evaporation of the solvent from this extract gave a glassy yellow substance which failed to crystallize, but which was chromatographically quite pure. The R_F's are given in Table I. The compound was obtained crystalline by acidification of a concentrated solution of it in aqueous $NaHCO_3$, but, because of a considerable solubility in water, the yield was poor. Like di-DNP-tabtoxinine (3), it tended to liquefy when in contact with water unless it was kept cold. When so obtained and dried at 68°, it melted at 111–115°. The yields of the compound extracted by ethyl acetate were

145 and 168 mg. in two experiments. The dried crystallized compound gave difficulty in the elementary analysis and it was necessary to burn it slowly.

$C_{16}H_{20}O_{11}N_4$. Calculated, C 43.3, H 4.51; found, C 43.6, H 4.35

DNP Toxin—The usual conditions for the preparation of DNP-amino acids could not be used because of the instability of the toxin to weak alkali. Consequently, 50 mg. of toxin were dissolved in 5 cc. of 0.5 M sodium phosphate at pH 7.5. The DNFB (300 mg. in 10 cc. of ethanol) was added, and the mixture was shaken vigorously for 3 hours. The manipulations were then the same as those described above, except for the following. The DNP toxin was unusually soluble in water, and not extractable from aqueous solution with ethyl acetate. It was therefore necessary to extract it with *n*-butanol immediately after the ethyl acetate extraction step. The butanol extract was washed once with water and freed of butanol by evaporation at 37° under reduced pressure in the presence of excess water. It was then dried and separated from traces of inorganic salts by extraction with acetone. The acetone-soluble residue was made to solidify by rubbing under benzene. The yield was 39 mg. R_F's are shown in Table I. Like the DNP-alkali-inactivated toxin, this compound also was difficult to burn satisfactorily.

$C_{16}H_{18}O_{10}N_4$. Calculated, C 45.0, H 4.2; found, C 45.1, H 4.4

This substance was rather unstable, and, when stored, slowly darkened and gave a variety of substances which could be separated on a paper strip developed with Solvent C.

DNP-Alkali-Inactivated Toxin from DNP Toxin—2 mg. of DNP toxin were dissolved in 4 cc. of M $NaHCO_3$ and, after the solution had stood overnight, it was acidified to pH 2 with HCl, and extracted with ethyl acetate four times. The extract contained about half of the original color. The R_F in Solvent C was 0.67, and in Solvent D 0.71. These values were the same as for an authentic sample of DNP-alkali-inactivated toxin run alongside. The extractability by ethyl acetate was the best distinction from DNP toxin.

ε-DNP-tabtoxinine—84 mg. of DNP-alkali-inactivated toxin were suspended in 15 cc. of 6 N HCl which was refluxed for 4 hours. Small amounts of decomposition products were removed by extraction with ethyl acetate and by filtration. The residual aqueous solution was concentrated to dryness under reduced pressure, and excess acid was removed in the usual way by repeated evaporations and by drying to constant weight *in vacuo* over KOH. The residue (40 mg.) was dissolved in 2 cc. of ethanol, and the product was precipitated by the addition of isobutylamine (yield 16

mg.). The material so obtained was recrystallized from water by addition of ethanol, and dried at 78° *in vacuo*. R_F's are shown in Table I.

$C_{12}H_{16}O_9N_4$.	Calculated.	C 41.9, H 4.3, N 15.1
	Found.	" 41.8, " 4.6, " 14.9

When this substance was allowed to react with DNFB in the usual way, the product was found to be di-DNP-tabtoxinine (3). (The R_F in Solvent A was 0.64, and in Solvent B 0.17.) Authentic di-DNP-tabtoxinine showed the same values.

The position of the DNP group was established by the results of the reaction with periodate. This destroyed all trace of the substance having R_F values corresponding to those of the original, and gave rise to a small amount of a yellow material with R_F 0.60 in Solvent A. Presumably, this should have been the DNP derivative of glutamic acid-γ-semialdehyde, but this was not proved further.

α-Formyl-ε-DNP-tabtoxinine—14 mg. of ε-DNP-tabtoxinine were dissolved in 10 cc. of 98 per cent formic acid which was refluxed for 8 hours. The solvent was removed under reduced pressure and the process was repeated four times. The final product was suspended in 5 cc. of 0.1 N HCl, which was then extracted three times with ethyl acetate. The extracted material was freed of solvent and dissolved in 4 cc. of 0.1 N NH_4OH. This was done in order to hydrolyze some *O*-formyl derivative which paper chromatography had shown to be present. After the slightly alkaline solution had stood at room temperature for a few minutes, it was acidified with HCl and extracted with ethyl acetate. The extracted material was separated on paper sheets by chromatography in Solvent C. The main yellow band was cut out; the compound was eluted from it and extracted into ethyl acetate from the acidified eluates. The yield was 11.5 mg. The R_F values in Solvents B and C were the same as for DNP-alkali-inactivated toxin. For some time, this was taken as evidence that the two compounds were identical, but quantitative determinations of formic acid and of lactic acid clearly differentiated them.

Identification of Formic Acid in Hydrolysates of Toxin—When 60 mg. of toxin were heated in 10 cc. of 6 N H_2SO_4 for 20 hours in a stoppered flask, and the solution was steam-distilled according to the official method for determination of formic acid (7), 0.05 mmole of volatile acid was found. This was shown to be formic acid by estimation of Duclaux numbers and from reduction of mercuric chloride. The Alicino method for formyl groups (5) gave slightly higher values (see the introduction).

Identification of Lactic Acid—When either the toxin, the alkali-inactivated toxin, or the DNP-alkali-inactivated toxin was refluxed in 4 N HCl or in 1 N NaOH, and the acidic hydrolysate was extracted continuously with

ether for 48 hours, the extract contained a non-volatile acidic fragment amounting to 20 to 30 per cent of the weight of the toxin. This acid exhibited a spot of R_F 0.52 on paper strips developed with Solvent E of Table I, and sprayed with brom cresol green, as described by Lugg and Overell (8). Lactic acid run alongside also showed R_F 0.52. Before application to the paper, both the known and the unknown were heated in 0.1 N NaOH in order to hydrolyze lactyllactic acid. In a solvent made from equal volumes of benzene, formic acid, ethanol, and water, equilibrated 3 days before separation of the phases, both lactic acid and the unknown acid showed a spot of R_F 0.43.

For the isolation of a crystalline derivative, it was found better to hydrolyze the toxin with alkali rather than with acid because the hydrolysates made with acid contained some substance which interfered with the quantitative colorimetric estimation of lactic acid in the Barker and Summerson procedure (9). Such a colorimetric determination in the ether extract was advisable in order to calculate how much phenacyl bromide to employ for the preparation of a solid ester. Excess reagent was difficult to remove from the final ester.

20 mg. of toxin were dissolved in 10 cc. of 1 N NaOH, and the solution was heated at 100° for 1 hour. It was then acidified to pH 2 with H_2SO_4 and extracted continuously with ether for 48 hours. Analysis of the extract revealed 5.5 mg. of lactic acid. The extracted material was freed of ether, warmed in an excess of NaOH for 10 minutes at 100°, and the solution was adjusted to pH 8 and to 1 cc. A solution of 15 mg. of *p*-phenylphenacyl bromide in 2 cc. of ethanol was added, and the mixture was refluxed for 1 hour. The ester was precipitated by evaporation to 1 cc. and addition of 3 cc. of water. Material so obtained was dried and dissolved in 1 cc. of benzene containing 1 per cent of methanol. This was applied to a column of dry silica gel 1 × 22 cm. which had been poured in the same solvent. The first 45 cc. of effluent were discarded, and the solvent was changed to 2 per cent methanol in benzene. The first 80 cc. of the effluent from this solvent were discarded, and the next 10 cc. were collected. The solvents were removed from this fraction under reduced pressure and the white solid which remained was recrystallized from petroleum ether. The crystals so obtained melted at 135–140°. This melting point was not depressed by admixture with authentic *p*-phenylphenacyl lactate. The rate of flow of the ester on the silica gel column was the same as that found for the known *p*-phenylphenacyl lactate in the same solvent systems, and thus constituted an additional proof of the identity of the two substances. The elution from the columns was followed by evaporation of the solvents from each 5 cc. fraction, solution in methanol, and determination of adsorption at 280 mμ. The unknown, like the known, ester was eluted in a well separated peak.

When acid hydrolysates of the DNP-alkali-inactivated toxin were extracted continuously with ether, some yellow, neutral substances were present in the extract in addition to the lactic acid.

Quantitative Determination of Lactic Acid—1 mg. of the toxin was dissolved in 2 cc. of 0.1 N NaOH and the solution was held at 100° for 5 minutes. Aliquots were then taken for the procedure of Barker and Summerson (9). This determination indicated 0.39 mg. of lactic acid, or 39 per cent of the toxin. Theory for the proposed structure was 35 per cent.

For analysis of the DNP-alkali-inactivated toxin, it was necessary to extract the lactic acid after the alkaline hydrolysis. When the determination was attempted directly on the hydrolysate, no lactic acid was found. Therefore, after the hydrolysis with 1 N NaOH for 10 minutes at 100°, the solution was acidified with H_2SO_4 to pH 1 and extracted continuously with ether for 48 hours. The extract was treated with 1 cc. of 1 N NaOH, and the ether was evaporated. The residue was dried thoroughly *in vacuo* in order to remove the last traces of acetaldehyde which arose from oxidation of the ether during the extraction, and aliquots were analyzed for lactic acid. The amount found corresponded to 20.2 per cent of the weight of the DNP-alkali-inactivated toxin. Theory was 20.3 per cent lactic acid.

SUMMARY

The phytopathogenic toxin of *Pseudomonas tabaci* has been shown to be most probably the lactone of α-lactylamino-β-hydroxy-ϵ-aminopimelic acid. The lactone is formed between the hydroxyl group of the lactic acid residue and the α-carboxyl group of the diaminohydroxypimelic acid. Several degradation products and other derivatives of this toxin have been prepared and characterized. The structural analogy of this pathogenic agent to methionine has thus been verified. Previous work had shown that methionine was a specific antagonist of the toxin, and had led to the postulate that the toxin was a naturally occurring antimetabolite of methionine.

BIBLIOGRAPHY

1. Braun, A. C., *Proc. Nat. Acad. Sc.*, **36,** 423 (1950).
2. Woolley, D. W., Pringle, R. B., and Braun, A. C., *J. Biol. Chem.*, **197,** 409 (1952).
3. Woolley, D. W., Schaffner, G., and Braun, A. C., *J. Biol. Chem.*, **198,** 807 (1952).
4. Crumpler, H. R., and Dent, C. E., *Nature*, **164,** 441 (1949).
5. Alicino, J. F., *Ind. and Eng. Chem., Anal. Ed.*, **15,** 764 (1943).
6. Porter, R. R., and Sanger, F., *Biochem. J.*, **42,** 287 (1948).
7. Official and tentative methods of analysis, of the Association of Official Agricultural Chemists, Washington, 5th edition (1940).
8. Lugg, J. W. H., and Overell, B. T., *Australian J. Scient. Res., Series A*, **1,** 98 (1948).
9. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138,** 535 (1941).

HEXOKINASES OF SCHISTOSOMA MANSONI*

By ERNEST BUEDING and JOAN A. MacKINNON

(From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio, and from the Department of Pharmacology, School of Medicine, Louisiana State University, New Orleans, Louisiana)

(Received for publication, October 22, 1954)

Extensive studies have been carried out on the hexokinase of yeast (1, 2) and of mammalian brain (3–7), but little is known about this enzyme in invertebrate Metazoa. It was felt that the parasitic worm *Schistosoma mansoni* might offer an opportunity to obtain information about this problem because of the extremely rapid rate of glycolysis of schistosomes (8) and because hexokinase activity of homogenates of the worms is of a high order. The studies reported in this paper have revealed that *S. mansoni* contains at least four different hexokinases, each one of which is characterized by a marked substrate specificity.

Material and Methods

Adult schistosomes were obtained as in previous studies (8, 9). The worms were homogenized in an all-glass Potter-Elvehjem homogenizer in glycylglycine buffer (0.01 M, pH 7.5); 1 ml. of buffer was used per 100 worm pairs. The protein concentration of this homogenate varied between 6.5 and 7.5 mg. per ml. Although the small size and the limited availability of the organisms precluded the possibility of an extensive purification, a hexokinase preparation could be obtained by the following fractionation procedure: 0.2 ml. of a suspension of calcium phosphate gel (14 mg. of dry weight per ml.) was added to 2 ml. of homogenate. After stirring, the mixture was centrifuged, the residue was discarded, and to 1.7 ml. of the supernatant fluid, 0.34 ml. of the same gel was added with stirring. After centrifugation, the residue was washed first with 2 ml. of 0.05 M and then with 1 ml. of 0.5 M glycylglycine buffer (pH 7.5). The second eluate (protein concentration 0.17 to 0.26 mg. per ml.), subsequently referred to as Fraction H, had high hexokinase activity. Its specific activity was 15 to 20 times higher than that of the original homogenate. The phosphofructokinase and ATP[1]-splitting activities of this fraction were negligible.

* This investigation was supported by a research grant from the Division of Research Grants, National Institutes of Health, United States Public Health Service.

[1] In this paper the following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TPN, triphosphopyridine nucleotide.

XUM

This and all other fractionations, to be described in a later section of this paper, were carried out at low temperatures (0–4°).

For enzyme purification with the aid of alumina gel Cγ (10) (22 mg. of dry weight per ml.), male worms (homogenized in 0.01 M glycylglycine buffer, pH 7.5) were used exclusively. It was found that reproducible results could not be obtained with homogenates or extracts of worm pairs and that there were considerable qualitative and quantitative variations in the hexokinase activities of female worms.

Hexokinase activities were determined by three procedures which were similar to those employed by Slein, Cori, and Cori (3). In the first method the decrease in the free hexose concentration during the incubation period (10 minutes, 37°) was measured after precipitation of the proteins and phosphate esters with the aid of $Ba(OH)_2$ and $ZnSO_4$ (11, 12). Glucose, mannose, and glucosamine were determined according to Somogyi (13), fructose according to Roe (14) as modified by Higashi and Peters (15), and deoxyglucose according to Sols and Crane (6). Hexose utilization was calculated from the difference between the free hexose concentration of the control (initial) and the experimental (incubated) sample. Hexokinase activity was expressed in micromoles of free hexose utilized in 10 minutes at 37° per mg. of protein. The other two methods were based on the spectrophotometric determination of glucose-6-phosphate (3). Formation of this ester was measured by following the change in the optical density at a wave-length of 340 mμ as a result of the reduction of TPN, due to the oxidation of glucose-6-phosphate by the action of *Zwischenferment* (glucose-6-phosphate dehydrogenase). For the direct determination of glucokinase activity *Zwischenferment* and TPN were present in the reaction mixture and the rate of reduction of TPN was measured over a period of 5 minutes. Fructokinase and mannokinase activities were determined in the same manner, except that phosphoglucose isomerase or a mixture of phosphoglucose isomerase and phosphomannose isomerase was added, respectively. The blank or reference cell contained all the constituents of the experimental sample (TPN, *Zwischenferment*, and, as needed, phosphoglucose isomerase and phosphomannose isomerase) with the exception of the schistosome extract. In this manner correction was made for any hexokinase activity of the enzymes used in the assay system. For indirect (3) determination of hexokinase activity the reaction mixtures were deproteinized with perchloric acid (final concentration 3 per cent) before (initial sample) and after (experimental sample) incubation and centrifuged at 0°. Aliquots of the supernatant fluids were neutralized (pH 7.5) and centrifuged again. Hexose phosphate contained in a measured volume of the supernatant fluid was determined spectrophotometrically as described by Slein *et al.* (3). The initial sample served as a control for any hexokinase activity of the en-

zymes used in the assay system; usually reduction of TPN in the presence of the initial sample was negligible and never exceeded 15 per cent of the values observed with the experimental samples.

The final molar concentrations of the constituents of the reaction mixture were as follows: ATP 7×10^{-3}, $MgCl_2$ 1×10^{-2}, potassium glycylglycine buffer (pH 7.5) 5×10^{-2}. The molar concentration of fructose was 4×10^{-2}; that of the other hexoses varied between 1 and 2×10^{-3}. Optimal concentrations for ATP and $MgCl_2$ (see Table I) and the pH optima were similar with any of the five hexoses as substrate.

Protein concentration was determined by measuring the optical density of the sample (diluted appropriately) at 420 mμ in trichloroacetic acid (final concentration 30 per cent) with the aid of a Beckman spectrophotometer. *Zwischenferment* was prepared by extracting 65 gm. (wet weight) of a xylose-adapted yeast[2] with 0.1 M $NaHCO_3$ (saturated with 5 per cent CO_2-95 per cent nitrogen) for 5 hours at 37°. Thereafter the procedure of Kornberg (16) was followed without modification.

A fraction of an aqueous extract of rabbit muscle precipitated between 0.52 and 0.72 saturation with $(NH_4)_2SO_4$ served as a source of phosphoglucose isomerase.

Phosphomannose isomerase was prepared according to Slein (17). Fructose-1-phosphate was prepared according to Raymond and Levene (18) and according to Leuthardt *et al.* (19, 20). A commercial preparation of glucosamine hydrochloride (Nutritional Biochemicals Corporation) was purified by dissolving 10 gm. of this material in 20 ml. of hot water and suspending in this solution 1 gm. of Norit A. To the filtered solution 5 volumes of ethanol were added. After cooling, the crystals were collected by filtration and dried. Solutions of this preparation were neutralized to pH 7.0 before use. Stock solutions of ATP were prepared by treatment of an acid solution of the barium salt (Pabst Laboratories) with a cation exchange resin (Dowex 50), followed by neutralization to pH 7.0.

Results

Homogenates of schistosomes and purified extracts, obtained by fractionation of these homogenates with calcium phosphate gel (Fraction H), catalyzed the phosphorylation of glucose, fructose, mannose, glucosamine, and 2-deoxyglucose in the presence of ATP and of Mg. Dissociation constants of glucose, fructose, ATP, and Mg were considerably higher for the extract of the parasite than for brain hexokinase (Table I). Under the conditions of these experiments the utilization of glucose (or of fructose) was not sufficient to permit accumulation of inhibitory concentrations of

[2] Supplied through Dr. O. J. Lampen by Dr. A. Wiley, Sulfite Pulp Manufacturing Research League, Appleton, Wisconsin.

reaction products (ADP, glucose-6-phosphate). Another difference between the properties of brain hexokinase and the enzyme preparation of schistosomes was revealed by the effect of glucose-6-phosphate on their activity. While this ester inhibits the phosphorylation of glucose, fructose, and mannose by brain hexokinase (21, 5), it inhibited only glucose and

TABLE I

Dissociation Constants (K_m) for Phosphorylation of Glucose and Fructose by Purified Schistosome Extracts (Fraction H)

Compound	K_m, moles per l.	
	Schistosome extract	Brain hexokinase (Sols and Crane (4))
ATP	1.5×10^{-3}*	1.3×10^{-4}
$MgCl_2$	3×10^{-3}*	8×10^{-4}
Glucose	1.5×10^{-4}	8×10^{-6}
Fructose	4×10^{-3}	1.6×10^{-3}

* Identical values were obtained when glucose or fructose was used as the substrate.

TABLE II

Effect of Glucose-6-phosphate and of L-Sorbose-1-phosphate on Hexokinase Activities of Purified Schistosome Extract (Fraction H)

Glucose-6-phosphate	L-Sorbose-1-phosphate	Per cent inhibition of utilization of		
		Glucose	Fructose	Mannose
M	*M*			
1.25×10^{-3}		66	0	71
6×10^{-4}		51	0	54
2×10^{-4}		18	0	22
	2×10^{-3}	72	0	40
	1×10^{-3}	53	0	16
	3×10^{-4}	28	0	0

mannose phosphorylation by the schistosome preparation. On the other hand, fructose phosphorylation was not affected (Table II). Similarly, L-sorbose-1-phosphate (22) inhibited phosphorylation of glucose and mannose, but not that of fructose (Table II). These observations suggested the possibility of the presence in the worms of more than one hexokinase. This was indicated also by considerable difference in the relative rates of phosphorylation of glucose, fructose, and mannose by homogenates of different groups of worms. In homogenates of fifteen different groups of

worms the ratios of phosphorylation varied as follows: fructose to glucose, 0.46 to 3.92; mannose to glucose, 0.40 to 1.46; fructose to mannose, 0.93 to 3.46. More conclusive evidence bearing on this problem was obtained by fractionating homogenates of male schistosomes at low temperatures (0–4°) with alumina gel Cγ. Three of these procedures will be described in the following paragraphs.

The protein concentration of the homogenate was adjusted to 5 mg. per ml., and 0.09 volume of the alumina gel was added. The mixture was stirred and centrifuged. The residue was eluted with 0.5 volume of 0.06 M glycylglycine buffer (pH 7.5). This eluate will be referred to as Fraction G-1.

After adjusting the protein concentration of the homogenate to 4.3 mg. per ml., adsorption was carried out with 0.06 volume of the gel. The residue was washed once with 1 volume of water and then eluted with 0.4 volume of 0.1 M glycylglycine buffer (pH 7.5). This eluate will be referred to as Fraction M.

TABLE III

Hexokinase Activities of Fractions of Male Schistosome Homogenates

Fraction	Utilization* of				
	Glucose	Fructose	Mannose	Glucosamine	2-Deoxy-glucose
Unfractionated homogenate	0.87	1.11	0.65	0.32	0.56
G-1	5.8	0	0	0	0
F	0	7.1	0	0	0
M	0	0	2.6	0	0

* Micromoles of hexose utilized per mg. of protein.

To each ml. of the homogenate (protein concentration 5.1 mg. per ml.) 0.02 ml. of a 10-fold dilution (dry weight 2.2 mg. per ml.) of the gel was added with stirring. After centrifugation, the residue was discarded, and 0.16 volume of the same dilution of the gel was mixed with the supernatant fluid. The residue was washed with 1 volume of water and then eluted with 0.5 volume of 0.3 M glycylglycine buffer (pH 7.5). This eluate will be referred to as Fraction F.

Fraction G-1 catalyzed the phosphorylation of glucose, but not that of the other four hexoses. Fraction F contained fructokinase free of other hexokinase activities, while Fraction M reacted exclusively with mannose (Table III). None of these three kinases, specific for either glucose, fructose, or mannose, catalyzed the phosphorylation of glucosamine or of 2-deoxyglucose (Table III). Recently some properties of the enzyme catalyz-

ing the phosphorylation of glucosamine in schistosomes have been reported (23). It has not been possible to establish whether the enzyme reacts with 2-deoxyglucose or whether phosphorylation of this hexose is catalyzed by

TABLE IV

Effect of Hexoses on Activity of Schistosome Fructokinase (Fraction F)

Molar concentration of fructose 1.4×10^{-2}.

Hexose	Concentration	Fructokinase activity*	Per cent inhibition
	M		
		6.6	
Glucose	1×10^{-2}	2.0	70
"	2×10^{-3}	3.1	53
"	4×10^{-4}	4.8	27
"	1×10^{-4}	6.4	3
Mannose	1×10^{-2}	2.7	59
"	2×10^{-3}	4.1	38
"	4×10^{-4}	6.7	0
Glucosamine	1×10^{-2}	2.3	65
"	2×10^{-3}	3.6	45
"	4×10^{-4}	6.3	5
Galactose	1×10^{-2}	4.6	30
"	2×10^{-3}	6.7	0
"	4×10^{-4}	6.6	0

* As defined in the text.

TABLE V

Inhibition of Schistosome Glucokinase by ADP

ADP	ATP	Ratio, ATP:ADP	Per cent inhibition
M	M		
1.4×10^{-2}	1.4×10^{-2}	1	91
1.4×10^{-2}	7×10^{-3}	0.5	90
1.4×10^{-2}	3.5×10^{-3}	0.25	86
7×10^{-3}	1.4×10^{-2}	2	62
7×10^{-3}	7×10^{-3}	1	64
7×10^{-3}	3.5×10^{-3}	0.5	57
2.3×10^{-3}	1.4×10^{-2}	6.1	35
2.3×10^{-3}	7×10^{-3}	3.05	33
2.3×10^{-3}	2.3×10^{-3}	1	29

another kinase, possibly one with a substrate specificity similar to that of brain hexokinase.

In contrast to liver fructokinase (24), the fructokinase of schistosomes (Fraction F) did not catalyze the phosphorylation of L-sorbose. The ac-

tivity of the latter enzyme was inhibited by other hexoses. Among those tested, glucose was found to be the most potent inhibitor (Table IV). Slein *et al.* have found that glucose and mannose inhibit the phosphorylation of

TABLE VI

Effect of Glutathione on Inhibition of Schistosome Glucokinase by p-Chloromercuribenzoate (PCMB)

Solutions of schistosome glucokinase were incubated at room temperature in the presence of glucose without or with PCMB. After 15 minutes, a solution of glutathione (GSH) or water was added. Following a second incubation period of 15 minutes at room temperature, a solution containing ATP and $MgCl_2$ was added, as well as enough PCMB and GSH to make their concentration equal to that during the preceding incubation periods.

PCMB	Glutathione	Glucokinase activity*	Per cent inhibition
M	*M*	*M*	
		6.4	
	1×10^{-3}	6.7	
1×10^{-5}		3.1	52
1×10^{-5}	1×10^{-3}	5.9	12
2×10^{-5}		1.0	84
2×10^{-5}	1×10^{-3}	5.3	20

* As defined in the text.

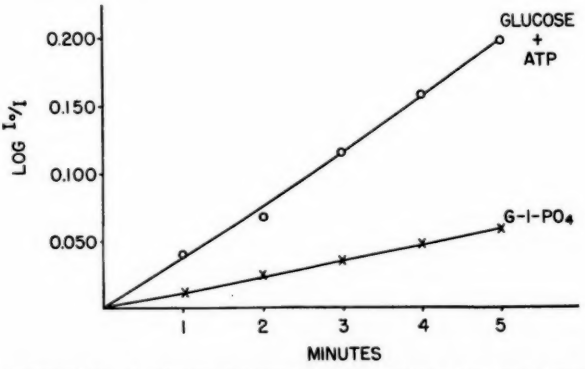


FIG. 1. Formation of glucose-6-phosphate determined by the direct spectrophotometric method. Fraction G-2 served as the source of glucokinase. The final molar substrate concentrations were as follows: glucose, 2×10^{-3}; ATP, 7×10^{-3}; glucose-1-phosphate, 2×10^{-3}.

fructose by the hexokinases of yeast and of brain, while these two hexoses do not affect the activities of the fructokinases of liver and of skeletal muscle (3).

Sols and Crane have demonstrated that adenosine diphosphate (ADP) is an inhibitor of brain hexokinase (6). Similarly ADP inhibited the activity of the glucokinase of schistosomes. However, unlike the case of brain hexokinase (6), inhibition of the worm enzyme by ADP is not reduced when the concentration of ATP is raised (Table V).

It appears that, as in the case of brain hexokinase (6), the hexokinases of schistosomes require intact sulfhydryl groups for their catalytic activities, because these enzymes are inhibited by low concentrations of mapharsen (25) and of other sulfhydryl inhibitors, such as iodoacetamide and *p*-chloromercuribenzoate. The inhibitory effects of the latter compound were prevented and reversed by glutathione (Table VI).

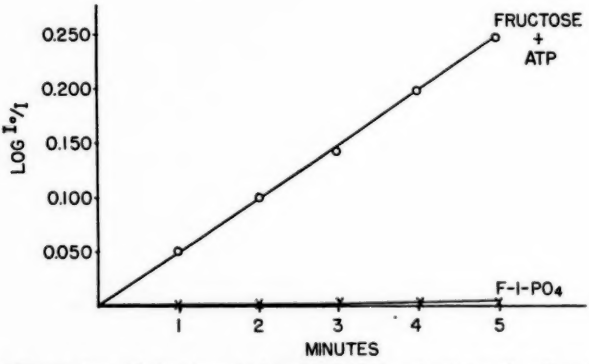


FIG. 2. Formation of fructose-6-phosphate determined by the direct spectrophotometric method. Fraction F served as the source of fructokinase. The final molar substrate concentrations were as follows: fructose, 1.4×10^{-2}; ATP, 7×10^{-3}; fructose-1-phosphate, 2×10^{-3}.

Observations obtained with both the direct and the indirect spectrophotometric procedures suggested that glucose-6-phosphate was the product of the reaction catalyzed by the schistosome glucokinase. However, since extracts of the worms contained high phosphoglucomutase activity, phosphorylation could have occurred in position 1, followed by a conversion of glucose-1-phosphate to glucose-6-phosphate through the action of phosphoglucomutase. This possibility was tested by the use of a glucokinase fraction which had low phosphoglucomutase activity. The extract was prepared as follows: The protein concentration of a homogenate of male worms was adjusted to 5 mg. per ml. and 0.04 volume of alumina gel Cγ was added with stirring. The mixture was centrifuged, and to the supernatant fluid another 0.04 volume of the gel was added. After centrifugation, the residue was eluted with 0.5 volume of 0.25 M and then with

0.5 volume of 0.5 M glycylglycine buffer (pH 7.5). With the latter eluate (referred to as Fraction G-2) the rate of formation of glucose-6-phosphate was considerably greater in the presence of glucose and ATP than in the presence of glucose-1-phosphate (Fig. 1). These observations rule out glucose-1-phosphate as the primary product of the reaction catalyzed by glucokinase of schistosomes.

Purified fructokinase of *S. mansoni* (Fraction F) did not catalyze the conversion of fructose-1-phosphate to fructose-6-phosphate (Fig. 2). Accordingly, it is concluded that schistosome fructokinase catalyzed the phosphorylation of fructose in position 6.

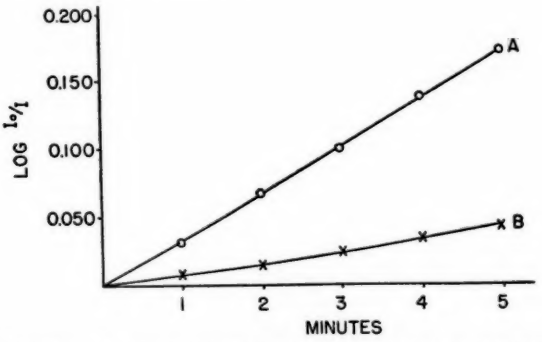


FIG. 3. Formation of mannose-6-phosphate determined by the direct spectrophotometric method. Fraction M was the source of the mannokinase. Curve A, phosphomannose isomerase (17) present in the assay system; Curve B, phosphomannose isomerase omitted from the assay system.

Direct and indirect spectrophotometric measurements of mannokinase activity revealed that Fraction M contained little phosphomannose isomerase activity. Reduction of TPN was considerably greater and agreed with the rate of mannose disappearance when phosphomannose isomerase of rabbit muscle was added to the assay system (Fig. 3).

DISCUSSION

Crystalline yeast hexokinase catalyzes the phosphorylation of glucose, fructose, mannose, glucosamine, and 2-deoxyglucose (1, 2, 26, 27). The same is true for purified brain hexokinase (3, 4, 7). By contrast, the hexokinases of *S. mansoni* are characterized by a much greater substrate specificity. The glucokinase and the fructokinase of the parasite catalyze phosphorylation in position 6 of the corresponding hexoses. The occurrence of specific glucokinases in skeletal muscle (3), in liver (3), and in some bacteria (28, 29) has been reported, but it is not known whether these en-

zymes catalyze the phosphorylation of glucose in position 1 or 6. Liver and muscle contain specific fructokinases (3, 18, 30–35). In contrast to the action of schistosome fructokinase, fructose-1-phosphate is the primary reaction product of mammalian fructokinases. To our knowledge, the occurrence of enzymes which specifically catalyze the transfer of phosphate from ATP either to mannose or to glucosamine has not been reported previously.

The activities of both brain hexokinase (6) and of schistosome glucokinase are inhibited by sulfhydryl inhibitors, by glucose-6-phosphate, by L-sorbose-1-phosphate, and by ADP. The two enzymes differ from each other by their substrate specificities and by their affinities for glucose, ATP, and Mg. Furthermore, while ATP competitively antagonized the inhibition by ADP of brain hexokinase (6), no such effect was observed with schistosome glucokinase.

Fructokinase of schistosomes differs from the fructokinases of liver and muscle because it catalyzes the phosphorylation of fructose in position 6, because its activity is inhibited by glucose and other hexoses, and because it does not react with L-sorbose. In these respects the schistosome enzyme resembles brain hexokinase. It is distinguished from the latter by its substrate specificity, by higher substrate-enzyme dissociation constants, and by the fact that glucose-6-phosphate and L-sorbose-1-phosphate do not inhibit its activities.

Differences in the nature and in the substrate specificities of hexokinases and of other enzymes catalyzing similar reactions in the parasite and in the host may afford opportunities to develop selective enzyme inhibitors. The occurrence in schistosomes of a specific glucosamine kinase and the observation that glucosamine-6-phosphate inhibits the glucokinase and the rate of glycolysis of the worms (23) suggested an investigation of the effect of glucosamine on the parasite. Such a study resulted in the finding that glucosamine reduces the survival of *S. mansoni in vitro* (23). The fact that glucosamine-6-phosphate has no inhibitory effect on brain hexokinase (36) might explain the low toxicity of glucosamine for the mammalian host.

We are indebted to Dr. H. A. Lardy for the L-sorbose-1-phosphate, to Dr. R. K. Crane for the 2-deoxyglucose, to Dr. B. Horecker for the glucose-6-phosphate, to Dr. E. W. Sutherland, Jr., for the glucose-1-phosphate, and to Dr. E. C. Ellingson for the fructose used in these experiments.

SUMMARY

1. *Schistosoma mansoni* contains four distinct hexokinases, each one of which reacts specifically with either glucose, fructose, mannose, or glucosamine.

2. Schistosome glucokinase catalyzes the phosphorylation of glucose in position 6. Fructose-6-phosphate was found to be the primary product of the reaction catalyzed by schistosome fructokinase.

3. As in the case of brain hexokinase, glucokinase of schistosomes is inhibited by glucose-6-phosphate, by sorbose-1-phosphate, by ADP, and by sulfhydryl inhibitors. The glucokinase of the parasite differs from brain hexokinase by its higher dissociation constants for glucose, ATP, and Mg as well as by its substrate specificity.

4. In contrast to fructokinase of mammalian liver and muscle, schistosome fructokinase is inhibited by glucose and does not catalyze the phosphorylation of L-sorbose.

BIBLIOGRAPHY

1. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29,** 379 (1946).
2. Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, **29,** 393 (1946).
3. Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **186,** 763 (1950).
4. Sols, A., and Crane, R. K., *Federation Proc.*, **12,** 271 (1953).
5. Crane, R. K., and Sols, A., *J. Biol. Chem.*, **203,** 273 (1953).
6. Sols, A., and Crane, R. K., *J. Biol. Chem.*, **206,** 925 (1954).
7. Sols, A., and Crane, R. K., *J. Biol. Chem.*, **210,** 581 (1954).
8. Bueding, E., *J. Gen. Physiol.*, **33,** 475 (1950).
9. Bueding, E., and Peters, L., *J. Pharmacol. and Exp. Therap.*, **101,** 210 (1951).
10. Bauer, E., in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, Leipzig, 1449 (1949).
11. Somogyi, M., *J. Biol. Chem.*, **160,** 69 (1945).
12. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **168,** 583 (1947).
13. Somogyi, M., *J. Biol. Chem.*, **160,** 61 (1945).
14. Roe, J. H., *J. Biol. Chem.*, **107,** 15 (1934).
15. Higashi, A., and Peters, L., *J. Lab. and Clin. Med.*, **35,** 475 (1950).
16. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
17. Slein, M. W., *J. Biol. Chem.*, **186,** 753 (1950).
18. Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83,** 619 (1929).
19. Leuthardt, F., and Testa, E., *Helv. chim. acta*, **34,** 931 (1951).
20. Leuthardt, F., Testa, E., and Wolf, H. P., *Helv. chim. acta*, **36,** 227 (1953).
21. Weil-Malherbe, H., and Bone, A. D., *Biochem. J.*, **49,** 339 (1951).
22. Lardy, H. A., Wiebelhaus, V. D., and Mann, K. M., *J. Biol. Chem.*, **187,** 325 (1950).
23. Bueding, E., Ruppender, H., and MacKinnon, J., *Proc. Nat. Acad. Sc.*, **40,** 773 (1954).
24. Leuthardt, F., and Testa, E., *Helv. physiol. et pharmacol. acta*, **8,** C67 (1950).
25. Mansour, T. E., and Bueding, E., *Brit. J. Pharmacol.*, **9,** 459 (1954).
26. Brown, D. H., *Biochim. et biophys. acta*, **7,** 487 (1951).
27. Cramer, F. B., and Woodward, G. E., *J. Franklin Inst.*, **253,** 354 (1952).
28. Cardini, C. E., *Enzymologia*, **14,** 362 (1951).
29. Klein, H. P., and Doudoroff, M., *J. Bact.*, **59,** 739 (1950).
30. Cori, C. F., *Biol. Symposia*, **5,** 131 (1941).
31. Leuthardt, F., and Testa, E., *Helv. chim. acta*, **33,** 1919 (1950).
32. Staub, A., and Vestling, C. S., *J. Biol. Chem.*, **191,** 395 (1951).

XUM

33. Cori, G. T., Ochoa, S., Slein, M. W., and Cori, C. F., *Biochim. et biophys. acta*, **7**, 304 (1951).
34. Hers, H. G., *Biochim. et biophys. acta*, **8**, 416 (1952).
35. Hers, H. G., *Arch. internat. physiol.*, **61**, 426 (1953).
36. Crane, R. K., and Sols, A., *J. Biol. Chem.*, **210**, 597 (1954).

STUDIES OF THE PHOSPHOGLUCOSE ISOMERASE OF SCHISTOSOMA MANSONI*

By ERNEST BUEDING and JOAN A. MacKINNON

(From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio, and from the Department of Pharmacology, School of Medicine, Louisiana State University, New Orleans, Louisiana)

(Received for publication, October 22, 1954)

Survival and reproduction of the parasitic worm *Schistosoma mansoni* are dependent on a high rate of anaerobic carbohydrate metabolism (1, 2). In contrast to *Ascaris* (3) and to other helminths (4), in schistosomes lactic acid is the end-product of fermentation. In this respect these organisms resemble the tissues of their host. As in vertebrate tissues, lactic acid is formed by *S. mansoni* via the Embden-Meyerhof scheme (5). These biochemical similarities would preclude the possibility of interfering with the carbohydrate metabolism of the parasite without injury to the host if the glycolytic enzymes catalyzing the same reactions in schistosomes and in mammalian tissues were identical. This problem was studied by comparing the properties of phosphoglucose isomerase of *S. mansoni* with those of rabbit muscle. Although these two enzymes have many characteristics in common, it was found that they are not identical with each other.

EXPERIMENTAL

Assay of Enzymatic Activity—The rate of formation of glucose-6-phosphate from fructose-6-phosphate catalyzed by phosphoglucose isomerase was followed spectrophotometrically. The oxidation of glucose-6-phosphate, catalyzed by *Zwischenferment*, was determined by measuring, for a period of 4 minutes, the increase in the optical density at 340 mμ as a result of the reduction of triphosphopyridine nucleotide (TPN). In every experiment correction for the slight isomerase activity of the *Zwischenferment* preparation was made by simultaneous determination of the blank TPN reduction of the assay system without added schistosome (or rabbit muscle) isomerase. The assay system contained per ml. 0.05 Kornberg unit (6) of *Zwischenferment*, 8×10^{-4} mmole of fructose-6-phosphate, 1.5×10^{-2} mmole of $MgCl_2$, 0.03 mmole of glycylglycine buffer (pH 9.0), and 1.5×10^{-5} mmole of TPN. 1 unit of phosphoglucose isomerase is defined as the amount of enzyme which brings about the reduction of 1 mμmole of TPN per minute.

* This investigation was supported by a research grant from the Division of Research Grants, National Institutes of Health, United States Public Health Service.

Zwischenferment was prepared as described previously (7). A fraction of an aqueous extract of rabbit muscle precipitated between 0.52 and 0.72 saturation with $(NH_4)_2SO_4$ served as the source of mammalian phosphoglucose isomerase (8).

Fructose-6-phosphate was purified as follows: To 400 mg. of a commercial sample of the barium salt (Nutritional Biochemicals Corporation) in 4 ml. of water were added 150 mg. of Nuchar C-N. After stirring, the mixture was filtered through a sintered glass funnel. The colorless solution was seeded with a few crystals of glucose-6-phosphate[1] and was allowed to remain in a refrigerator for 6 days. The crystalline precipitate of glucose-6-phosphate was centrifuged; the supernatant solution was treated with a cation exchange resin (Dowex 50) and neutralized to pH 7.0. This solution contained traces of glucose-6-phosphate, and interference of this ester with the assay was eliminated by incubating the reaction mixture for 10 minutes before addition of the isomerase. During this period oxidation of the glucose-6-phosphate present in the reaction mixture was completed. Crystalline glucose-6-phosphate (barium salt) was kindly supplied by Dr. B. L. Horecker.

Purification of Phosphoglucose Isomerase of S. mansoni—Worm pairs of schistosomes were placed in potassium glycylglycine buffer (0.01 M, pH 7.5) (1 ml. of buffer per forty pairs), cut with fine scissors for 2 minutes, stirred for 5 minutes, and then centrifuged for 10 minutes at 4000 r.p.m. 320 mg. of ammonium sulfate were dissolved per ml. of extract, and the mixture was centrifuged for 15 minutes at 7000 r.p.m. To each ml. of the resulting supernatant fluid 300 mg. of ammonium sulfate were added with stirring; the mixture was centrifuged for 15 minutes at 10,000 r.p.m., and the residue was dissolved in a volume of sodium glycylglycine buffer (0.025 M, pH 7.5) equivalent to one-fourth of the original worm extract. To this solution 0.04 volume of a suspension of calcium phosphate gel (14 ml. of dry weight per ml.) (9) was added with stirring. The suspension was centrifuged, and the supernatant fluid was used as the source of the enzyme. All operations were carried out at temperatures varying between 0–4°. Protein concentrations were determined by measuring the optical density of the sample (diluted appropriately) at 420 mμ in trichloroacetic acid (final concentration 30 per cent) with the aid of a Beckman spectrophotometer.

The activities of fourteen such preparations varied between 1200 and 2000 isomerase units per ml.; their protein concentration varied between 0.12 and 0.19 mg. per ml. On the basis of the specific activities of the original extracts, this procedure resulted in a 20- to 30-fold purification. When stored in the frozen state, the activities of the purified preparation remained constant for a period of at least 10 days.

[1] B. L. Horecker, personal communication.

Kinetics of Isomerases of Schistosomes and of Rabbit Muscle—The ratios of glucose-6-phosphate to fructose-6-phosphate at equilibrium were determined by procedures described by Somers and Cosby (10). Fructose was determined according to Roe (11), as modified by Higashi and Peters (12). Isomerase (500 units) was present in a total volume of 1.25 ml. The initial concentration of the substrate was 0.03 M. Equilibrium was reached in less than 5 minutes (37°), and the values obtained were identical when either glucose-6-phosphate or fructose-6-phosphate was used as the substrate.

The kinetics of the isomerases of rabbit muscle and of *S. mansoni* revealed close similarities in the properties of these two enzymes (Table I). The dissociation constants for fructose-6-phosphate, as well as the equilibrium constants, were identical. Optimal activity of both enzymes was observed at pH 8.6, but, in the case of the worm enzyme, the optimum extended to pH 8.2, while the activity of the mammalian enzyme was slightly, but significantly, reduced at this lower pH.

TABLE I

Kinetics of Phosphoglucose Isomerases of Rabbit Muscle and of S. mansoni

	Rabbit muscle enzyme	Worm enzyme
Optimal pH	8.6	8.2–8.6
K_m, fructose-6-phosphate	1×10^{-4}	1×10^{-4}
Ratio, glucose-6-phosphate to fructose-6-phosphate at equilibrium	65:35	65:35

The close kinetic similarities did not prove definitively that the two enzymes are identical. Evidence bearing on this question could be obtained by the use of an antibody against one of these enzymes, because differences in their interaction with such antibodies would reveal differences in their nature. On the other hand, if the two enzymes were identical with each other, they should exhibit similar behavior toward specific antibodies. Therefore, an attempt was made to obtain such an antibody.

Preparation and Properties of Immune Serum against Phosphoglucose Isomerase of Schistosomes—To a rooster, weighing 1.8 kilos, seven doses of purified phosphoglucose isomerase were administered. 1.4 to 2.7 ml. of the enzyme solution in a suspension of alum (13) were used per injection. The first three doses were given every other day, and four additional doses were administered 1, 3, 5, and 7 weeks after the third injection. The animal received a total of 15,900 units of the enzyme. The total amount of protein administered was 3 mg. (approximately 0.45 mg. of protein per injection). The serum which was collected 1 week after the last injection

will be referred to as immune serum. Its inhibitory effect on the schistosome isomerase remained constant during storage in the frozen state for a period of at least 3 months.

Before incubation of the enzyme with the serum, the latter was heated for 30 minutes at 55°. In addition to the inactivation of complement, this procedure almost completely abolished the isomerase activity of the serum, but resulted in no reduction of its inhibitory activity on the worm isomerase. The enzyme solution (diluted with 0.025 M glycylglycine buffer, pH 7.5) was added to the control or to the immune serum or to a dilution thereof, and the mixture was allowed to stand for 10 minutes at room temperature in a total volume of 0.08 ml. Unless stated otherwise, the concentration

TABLE II

Effect of Sera on Activities of Phosphoglucose Isomerase of Schistosomes and of Rabbit Muscle

All activities are expressed in millimicromoles of TPN reduced per minute; inhibition, in per cent.

Serum added	*S. mansoni*		*S. japonicum*		*S. hematobium*		Rabbit muscle	
	Activity	Inhibition	Activity	Inhibition	Activity	Inhibition	Activity	Inhibition
None	2.7		3.1		4.0		3.5	
Control*	2.7	0	3.1	0	3.8	0	3.3	6
Immune*	1.3	53	1.3	58	1.6	60	3.4	3
Antiserum* against schistosome lactic dehydrogenase	2.8	0						

* Corrected for blank activity of serum.

of the serum was 25 per cent. At the end of the incubation period a solution containing all the other constituents of the assay mixture was added, and spectrophotometric measurement of enzymatic activity was begun. In the calculation of the latter, correction for the small blank isomerase activity of the serum was made which was determined in a control sample, in the absence of added enzyme. This activity never was greater than one-tenth that of the solution of worm isomerase used in the assay.

The immune serum markedly inhibited the activity of the phosphoglucose isomerases of *S. mansoni*, *Schistosoma japonicum*, and of *Schistosoma hematobium* (Table II). By contrast, the immune serum did not affect the activity of isomerase of rabbit muscle. The serum of the same rooster obtained before the administration of the worm enzyme (subsequently referred to as the control serum) and serum of untreated roosters had no effect on the activities of the schistosome isomerases. Similarly, enzy-

matic activity was not inhibited by an antiserum against lactic dehydrogenase of *S. mansoni* (14) (Table II).

In the experiments to be described below the inhibitory effect of the immune serum was calculated by the use of the following equation

$$\% \text{ inhibition} = \frac{A_C - A_I}{A_C} \times 100$$

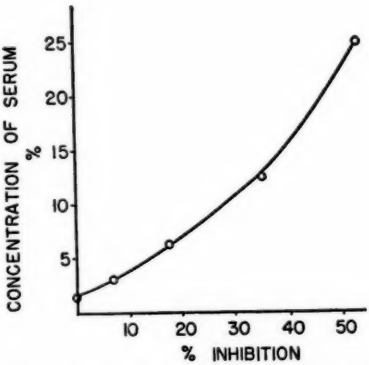


FIG. 1. Effect of concentration of immune serum on the activity of phosphoglucose isomerase of *S. mansoni*.

TABLE III

Effect of Preincubation of Schistosome Isomerase with Fructose-6-phosphate or with Glucose-6-phosphate on Inhibitory Action of Immune Serum

Enzyme preincubated with	Per cent inhibition
Buffer	47
Fructose-6-phosphate (8×10^{-3} M*)	30
" (1.6×10^{-2} M*)	14
Glucose-6-phosphate (1×10^{-2} M*)	21

* Final concentration during preincubation.

where A_C and A_I are the activities of the enzyme in the presence of the control and of the immune serum, respectively. The inhibitory effect of the immune serum on the worm isomerase was diminished markedly as the concentration of the serum during incubation with the enzyme was lowered (Fig. 1). When the schistosome isomerase was incubated with fructose-6-phosphate prior to the addition of the immune serum, the inhibitory effect of the latter was reduced (Table III). Similarly, glucose-6-phosphate

afforded partial protection from the inhibitory effect of the immune serum (Table III).

DISCUSSION

The observations reported in this study indicate that, in spite of close kinetic similarities, enzymes catalyzing the same reaction in the parasite and in the host are not necessarily identical with each other. As is the case with other proteins, enzymes, apart from their substrate specificities, are characterized by species specificities. It appears that differences between the enzyme of the host and of the parasite may involve their active centers because the substrate affords partial protection of the worm isomerase from inhibition by the immune serum. The properties of the latter revealed the possibility that the functional integrity of a schistosome enzyme can be inhibited without affecting the enzyme, which has the same function in the host. An antiserum against lactic dehydrogenase of *S. mansoni* (14) did not affect the activity of phosphoglucose isomerase of the same parasite; conversely, the immune serum against the latter enzyme had no effect on lactic dehydrogenase of schistosomes (14). Because of this complete absence of cross-reactions, it is evident that the two antisera react with sites specific for a particular enzyme of the parasite, rather than with groupings common to schistosome proteins.

While the phosphoglucose isomerase of schistosomes could be distinguished from that of rabbit muscle only by a specific antibody, the lactic dehydrogenases of schistosomes and of rabbit muscle differ from each other by their affinity for one of their substrates and by the effect of pH on their activities (15). In the case of the hexokinases of mammalian tissues and of *S. mansoni* differences in their kinetics as well as in their substrate specificities were observed (5). Therefore, differences between the enzymes of the parasite and of the host may occur at various levels.

SUMMARY

Phosphoglucose isomerase of *Schistosoma mansoni* was purified, and its properties were compared with those of phosphoglucose isomerase of rabbit muscle. In spite of close kinetic similarities, the two enzymes are not identical with each other. A specific antiserum, produced by immunizing a rooster against the schistosome isomerase, inhibited the activity of this enzyme, but did not affect that of the rabbit muscle isomerase. Fructose-6-phosphate and glucose-6-phosphate partially protected the schistosome enzyme from the inhibitory effects of the immune serum.

BIBLIOGRAPHY

1. Bueding, E., *J. Gen. Physiol.*, **33**, 475 (1950).
2. Bueding, E., and Peters, L., *J. Pharmacol. and Exp. Therap.*, **101**, 210 (1951).

3. Bueding, E., *J. Biol. Chem.*, **202,** 505 (1953).
4. Bueding, E., *Physiol. Rev.*, **29,** 195 (1949).
5. Bueding, E., and Most, H., *Ann. Rev. Microbiol.*, **7,** 295 (1953).
6. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
7. Bueding, E., and MacKinnon, J. A., *J. Biol. Chem.*, **215,** 495 (1955).
8. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173,** 605 (1948).
9. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B,* **124,** 397 (1938).
10. Somers, G. F., and Cosby, E. L., *Arch. Biochem.*, **6,** 295 (1945).
11. Roe, J. H., *J. Biol. Chem.*, **107,** 15 (1934).
12. Higashi, A., and Peters, L., *J. Lab. and Clin. Med.*, **35,** 475 (1950).
13. Mansour, T. E., Bueding, E., and Stavitsky, A. B., *Brit. J. Pharmacol.*, **9,** 182 (1954).
14. Henion, W., Mansour, T. E., and Bueding, E., *Exp. Parasitol.*, **4,** 40 (1955).
15. Mansour, T. E., and Bueding, E., *Brit. J. Pharmacol.*, **8,** 567 (1953).

(*From*

Pho
of rib
sen (3
pable
form,
of th
studie
there
is pre

Spe
Inc.,
Incor
and c
pared
0.067
centri
was p
used
(R-5-
cordi
from
phosp

* A
Biolog
(2).
† P
Servic
from
Scienc
for th
‡ P
Reser

XUM

STUDIES ON PHOSPHOMUTASES

II. PHOSPHORIBOMUTASE AND PHOSPHOGLUCOMUTASE*

By ARMAND J. GUARINO† and HENRY Z. SABLE‡

(From the Department of Biochemistry and Nutrition, Tufts College Medical School, Boston, Massachusetts)

(Received for publication, December 4, 1954)

Phosphoribomutase is the enzyme which catalyzes the interconversion of ribose-1-phosphate and ribose-5-phosphate. Recently, Klenow and Larsen (3, 4) reported that phosphoglucomutase of muscle and yeast is capable of catalyzing the conversion of ribose-1-phosphate to an acid-stable form, presumably ribose-5-phosphate. They made no mention, however, of the possible existence of a separate phosphoribomutase. We have studied extracts of yeast and of various mammalian tissues, and find that there may indeed be separate enzymes. Evidence supporting this view is presented in this paper.

Materials

Special active dried brewers' yeast was purchased from Anheuser-Busch, Inc., and *bakers' yeast* (*Fleischmann's*) was obtained from Standard Brands Incorporated as the freshly prepared pressed cake. This was then crumbled and dried at room temperature for 3 days. Maceration juice was prepared from either brewers' or bakers' yeast by autolysis in 3 volumes of 0.067 M dipotassium phosphate at 37° for 4 hours. Solids were removed by centrifugation at high speed in an angle centrifuge. *Calcium phosphate gel* was prepared by the method of Keilin and Hartree (5). *Guanosine* was used as obtained from the Schwarz Laboratories, Inc. *Ribose-5-phosphate* (R-5-P) was prepared by acid hydrolysis of adenosine triphosphate according to LePage and Umbreit (6), and *α-glucose-1-phosphate* (G-1-P) from potato starch by incubation with inorganic phosphate and potato phosphorylase. The glucose-1-phosphate was isolated as the barium

* A preliminary report of this work was presented before the American Society of Biological Chemists, April, 1954 (1). For Paper I of this series, see Sable and Calkins (2). Supported in part by a research grant from the Nutrition Foundation, Inc.

† Predoctoral Research Fellow of the National Cancer Institute, Public Health Service, 1952–53; Postdoctoral Research Fellow, 1953–55. Some of the data are taken from a dissertation presented by A. J. Guarino to the Graduate School of Arts and Sciences of Tufts College in August, 1953, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

‡ Present address, Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio.

XUM

salt; this was converted to the dipotassium salt and recrystallized three times.[1] *α-Glucose-1,6-diphosphate* (GDP) (7) was a gift of Dr. Theodore Posternak, and ethylenediaminetetraacetic acid (Versene, analytical grade disodium salt) was a gift of the Bersworth Chemical Company. *Bovine uterus* was purchased from the New England Dressed Meat and Wool Company. *Phosphoglucomutase* of rabbit muscle was prepared as described by Najjar (8), *nucleoside phosphorylase* of rat liver by the method of Kalckar (9), and *ribose-1-phosphate* (R-1-P) by a modification of the procedure of Dr. Graham T. Webster.[2] 10 ml. of the nucleoside phosphorylase of rat liver obtained as described were added to a solution of 2 gm. of guanosine in 700 ml. of 0.2 M potassium phosphate buffer, pH 6.8, and the mixture was incubated for 2½ hours at 37°. The reaction was stopped by immersing the reaction flask in a boiling water bath with vigorous stirring until the inner temperature reached 80°. The flask was immediately placed in an ice bath, and stirring was continued until the temperature fell to 4°. The mixture was stored at 2° overnight to allow complete precipitation of protein and guanine. A gelatinous precipitate formed, which was filtered readily on fluted paper. To the filtrate were added 175 ml. of M $MgCl_2$, followed by aqueous ammonia to bring the pH to 7.7. The mixture was left at room temperature for 4 hours and then filtered. The filtrate usually contained very little inorganic phosphate. At this stage of the procedure, 2 to 3 mmoles of ultralabile[3] organic phosphate were usually present in a volume of approximately 850 ml. This solution was concentrated under reduced pressure to approximately 100 to 150 ml. at a bath temperature not exceeding 40°. Excess calcium acetate was added, followed by absolute ethanol to give a final concentration of 70 per cent ethanol. The mixture was allowed to stand overnight. The precipitate was then collected by centrifugation and dried *in vacuo* over NaOH and paraffin.[4]

The dry calcium salt of R-1-P was suspended in approximately 25 ml. of water (dissolution was incomplete), excess potassium oxalate was added, and the mixture triturated. The precipitated calcium oxalate was removed by centrifugation and washed twice with small portions of water containing potassium oxalate, the washings being added to the original supernatant solution. To this solution an equal volume of absolute ethanol was added slowly to bring the ethanol concentration to 50 per cent. The

[1] The details of this procedure were supplied by Dr. E. W. Sutherland.

[2] Personal communication, unpublished experiments carried out at Washington University, 1950.

[3] This term is used to describe phosphate compounds which are hydrolyzed by acid at room temperature so rapidly that their phosphate appears to be inorganic under the usual conditions of the Fiske-Subbarow method.

[4] In several preparations barium acetate was used. This generally led to difficulties because of the insolubility of barium chloride in ethanol.

precipitate which formed was removed by centrifugation and discarded. The supernatant solution was then adjusted to 75 per cent ethanol concentration again by the slow addition of absolute ethanol. Too rapid addition of alcohol at this stage caused the potassium R-1-P to separate as an oil. The precipitated potassium salt was left at room temperature overnight. The supernatant solution was discarded and the crystalline precipitate dried *in vacuo* over NaOH and paraffin. This precipitate was usually dissolved in 10 to 20 ml. of water, and could be recrystallized in between 50 and 75 per cent ethanol if desired.

Analysis for inorganic phosphate, acid-labile phosphate, total phosphate, and pentose indicated that all the organically bound phosphate present is acid-labile and occurs as ribose-1-phosphate.[5] The extreme acid lability of R-1-P was described by Kalckar (9) and Lowry and Lopez (10), and we have confirmed this with a highly purified preparation. R-1-P is completely hydrolyzed by 0.5 N H_2SO_4 at 26° in less than 10 minutes; consequently it may be determined as apparent inorganic phosphate by the Fiske and Subbarow procedure (11), with appropriate corrections for true inorganic phosphate.

Because of the labor involved and the poor yields in this process, some of the experiments, particularly the earlier ones, were carried out with only partially purified R-1-P which had not been carried through the alcohol fractionation and which sometimes contained appreciable amounts of inorganic phosphate and some KCl. In the experiments in which the need for magnesium ions was investigated, the solution of the dipotassium salt was poured over a column of Amberlite IR-120 charged with potassium ions to insure complete removal of any divalent cations which might have remained in the preparation. In another experiment in which the rôle of Mg^{++} was examined, the crystalline cyclohexylamine salt of ribose-1-phosphate was used.

Some experiments were also carried out with material purified by ion exchange chromatography on Dowex 1 resin in the formate cycle according to Rowen and Kornberg (12).

Methods

Inorganic phosphate was determined by King's modification (13) of the Fiske-Subbarow (11) procedure. Inorganic phosphate in the presence of acid-labile phosphate was determined by the method of Lowry and Lopez (10). Acid-labile phosphate was determined by measuring the increase in inorganic orthophosphate after 10 minutes hydrolysis in normal sulfuric acid at 100°. Ultralabile phosphate[3] was measured as apparent inorganic

[5] Experiments on the structure of ribose-1-phosphate are in progress and will be reported at a later date.

orthophosphate corrected for the true inorganic orthophosphate. Pentose was determined by the method of Mejbaum (14), and protein by the method of Robinson and Hogden (15). All incubations were carried out at 30°.

RESULTS AND DISCUSSION

Yeast is known to have very active pentose-degrading systems (16) and nucleoside phosphorylases (17), and appeared likely to be a good source of

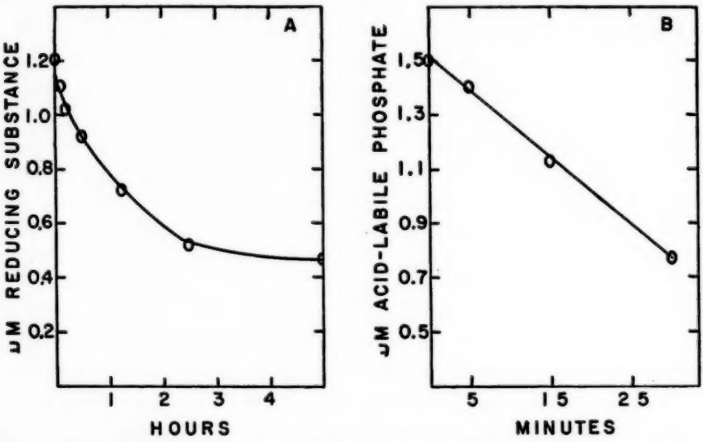


FIG. 1. Phosphoribomutase activity in a dialyzed extract of brewers' yeast. *A*, the replicate incubation mixtures contained, in a final volume of 1.2 ml., 1.2 μmoles of R-1-P, 0.009 M Tris (tris(hydroxymethyl)aminomethane) buffer, pH 7.6, 0.12 M NaF, and 0.2 ml. of yeast autolysate. Reactions were stopped by the addition of 0.2 ml. of N HCl, followed by N KOH, $Ba(OH)_2$, and $ZnSO_4$ (see the text). Reducing material was estimated in aliquots of the protein-free filtrates. *B*, the reaction mixtures contained, in a final volume of 1.2 ml., 1.5 μmoles of R-1-P, 0.02 M succinate-Tris buffer, pH 7.6, 0.01 M NaF. Reactions were started by the addition of 0.2 ml. of an autolysate of brewers' yeast (3.8 mg. of protein) and were stopped with perchloric acid. The protein-free filtrates were analyzed for acid-labile phosphate.

phosphoribomutase. Evidence for the occurrence of a mutase was obtained in two ways. First, the decrease in reducing substances not precipitated by the $Ba(OH)_2$-$ZnSO_4$ reagent of Somogyi (18) after a preliminary hydrolysis in N HCl at 30° (Fig. 1, *A*) was measured. In this procedure R-1-P is hydrolyzed and gives rise to free ribose in the filtrates, whereas R-5-P and other phosphate esters of ribose are not hydrolyzed and are adsorbed completely by the $Zn(OH)_2$ precipitate. Consequently the change in reducing power of the filtrates may be used as a measure of the conversion of R-1-P to another ester. By the second method of analysis, the conversion of acid-labile phosphate to an acid-stable form (Fig. 1, *B*) was measured.

In the experiments represented in Fig. 1, NaF was present in high concentrations. In other experiments not shown here, the omission of NaF had no effect on the rate of the reaction. This behavior is different from that observed with phosphoglucomutases (2, 8).

Dialyzed maceration juice of both bakers' and brewers' yeast was also examined for phosphoglucomutase, since this enzyme, if present, might be catalyzing the phosphoribomutase reaction. The conversion of acid-labile phosphate (G-1-P) to acid-stable phosphate (glucose-6-phosphate) was

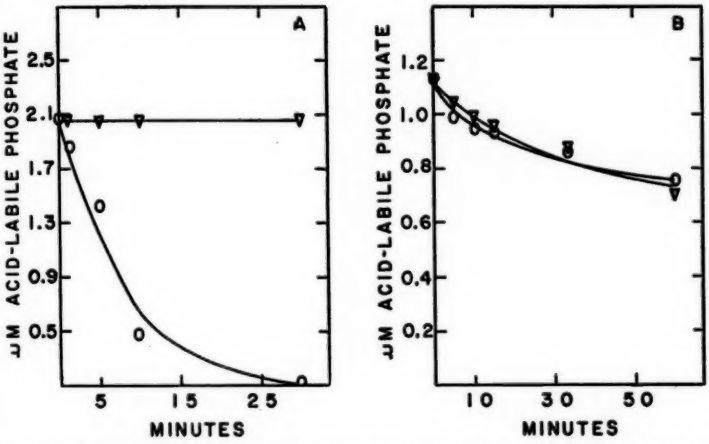


FIG. 2. Effect of Mg^{++} on phosphomutase activities in yeast extract. *A*, the reaction mixtures contained 2 μmoles of G-1-P, 0.03 M succinate-Tris buffer, pH 7.6, and 0.1 ml. of enzyme (Versene-treated, 1.1 mg. of protein). △, no added Mg^{++}; ○, 0.004 M $MgCl_2$ present. Reactions were stopped with perchloric acid, and the protein-free filtrates were analyzed for acid-labile phosphate. *B*, the reaction mixtures contained 1.1 μmoles of R-1-P, 0.015 M succinate-Tris buffer, pH 7.0, and 0.2 ml. of enzyme (Versene-treated, 2.2 mg. of protein). △, no Mg^{++} added; ○, 0.004 M Mg^{++} present.

measured. Substantial activity was found. In agreement with Klenow and Larsen (3, 4), we found that the ratio of phosphoglucomutase to phosphoribomutase activity in the autolysate was approximately 100:1.

In the experiments in which R-1-P was incubated with yeast extracts, no divalent cations were added. Phosphoglucomutase is inactive in the absence of divalent cations (8), and the apparent lack of such a requirement by phosphoribomutase was an unexpected finding. It was necessary to determine whether some divalent cation in the yeast extract was activating the enzyme.

10 ml. of yeast extract, after a preliminary dialysis overnight against 10 liters of distilled water, were treated with Versene (final concentration,

0.01 M), then dialyzed overnight against distilled water to remove any Versene-cation complex formed. This preparation was inactive in the phosphoglucomutase test unless magnesium ions were added (Fig. 2, *A*). On the other hand, the conversion of R-1-P to a stable ester proceeded equally well in the presence and the absence of added magnesium (Fig. 2, *B*), and even the presence of 0.01 M Versene in the incubation mixtures did

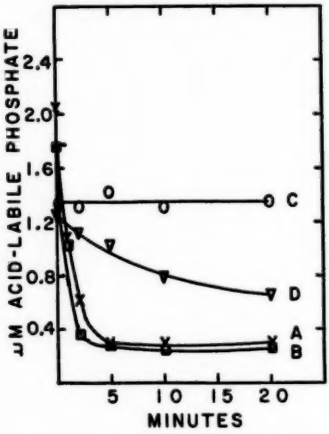


FIG. 3. Effect of heat on phosphoribomutase and phosphoglucomutase of bakers' yeast. Curves A and B, replicate reaction mixtures contained 2 μmoles of G-1-P, 0.03 M Tris buffer, pH 7.6, and 0.004 M $MgCl_2$ in a final volume of 1.1 ml. Curve A, the reactions were started with an unheated preparation of maceration juice (3.8 mg. of protein). Curve B, the reactions started with a heated preparation (0.84 mg. of protein) representing the same amount of yeast (see the text). Curves C and D, the reaction mixtures contained 1.2 μmoles of R-1-P, 0.03 M Tris buffer, pH 7.6, and 0.004 M $MgCl_2$. Curve D, the reactions started with unheated enzyme (3.8 mg. of protein). Curve C, the reactions were started with heated enzyme (0.84 mg. of protein). Reactions were stopped with perchloric acid and the protein-free filtrates analyzed for acid-labile phosphate.

not inhibit phosphoribomutase activity. This apparent difference in the requirement for magnesium ions was the first suggestion that two different enzymes might be involved in these reactions, and consequently an attempt was made to separate the two activities in the yeast extract.

A test-tube containing 10 ml. of maceration juice was placed in a boiling water bath, the contents were stirred until a temperature of 65° was reached, and the tube was then chilled in an ice bath to 4°. Denatured protein was removed by centrifugation and the supernatant solution analyzed for phosphoribomutase and phosphoglucomutase activities (Fig. 3). Phosphoglucomutase activity was essentially unimpaired by this treat-

ment, while phosphoribomutase activity was destroyed. In one experiment with such a heated extract, the lost phosphoribomutase activity was not restored by addition to the incubation mixture of 5×10^{-6} M GDP.

Attempts to separate the two activities by ammonium sulfate fractionation or isoelectric precipitation always resulted in complete loss of phosphoribomutase activity, while most of the phosphoglucomutase activity could still be recovered in the various protein fractions. These findings show that there is a separate phosphoribomutase which is considerably more labile than phosphoglucomutase under the conditions employed.

Various divalent cations were tested as possible activators of phosphoribomutase. The results of these experiments are reported in Table I.

TABLE I

Effect of Divalent Cations on Phosphoribomutase Activity of Yeast

Incubation mixtures contained 1.3 μmoles of R-1-P, 0.018 M succinate-Tris buffer, pH 7.0, 0.004 M divalent cation (chloride salt), and 0.2 ml. of yeast maceration juice (5 mg. of protein); final volume, 1.2 ml. Analyses were carried out as described in the legend for Fig. 1, *B*.

Ions present	Per cent inhibition
Mg^{++}	6
Ba^{++}	12
Mn^{++}	37
Co^{++}	53
Ni^{++}	57
Cu^{++}	100
Zn^{++}	100

As can be seen, none of the salts were stimulating and most were inhibitory at the concentration used (4×10^{-3} M). Complete inhibition was obtained with cupric and zinc ions, while cobalt, nickel, and manganous ions inhibited the activity to a variable extent. The inhibitory effects recorded for magnesium and barium ions are probably too small to be significant.

The pH optimum of bakers' yeast phosphoribomutase was found to lie between pH 6.7 and 7.2 (Fig. 4).

Smooth Muscle—Because of the lability of phosphoribomutase in yeast, another source was sought for the separation of this enzyme from phosphoglucomutase. Glycolytic reactions have been demonstrated in smooth muscle (19), but in general detailed studies have not been recorded. An investigation of the phosphomutases of this tissue seemed useful and interesting.

Definition of Unit Activity—In this investigation a unit of phospho-

mutase activity has been defined as the amount of enzyme which will catalyze the conversion of 0.1 μmole of acid-labile phosphate to an acid-stable form in 1 minute in the test system used.

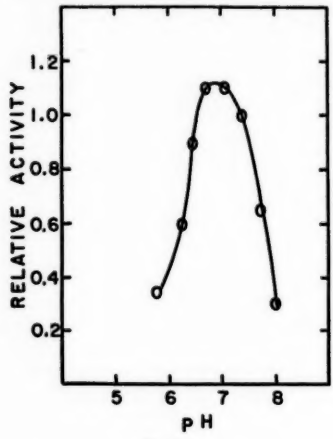


FIG. 4

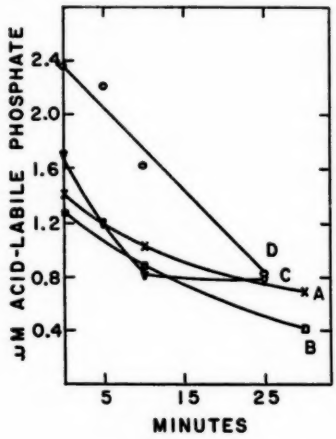


FIG. 5

FIG. 4. pH optimum of yeast phosphoribomutase. The reaction mixtures contained 1.1 μmoles of R-1-P, 0.005 M $MgCl_2$, 0.015 M succinate-Tris buffer, and 0.2 ml. of a dialyzed autolysate of bakers' yeast (4.2 mg. of protein). The reactions were stopped by perchloric acid, and aliquots of the protein-free filtrates analyzed for acid-labile phosphate. Units of activity (as defined in the text) are plotted on the ordinate. The actual pH of the incubated mixtures as measured with a glass electrode is indicated on the abscissa.

FIG. 5. Phosphomutase activity in rabbit and human uterus. Curve A, the reaction mixture contained 1.4 μmoles of G-1-P, 0.1 M Tris buffer, pH 7.6, 0.006 M $MgCl_2$, and 0.2 ml. of dialyzed extract of rabbit uterus (2.2 mg. of protein). Curve B, the reaction mixtures contained 1.3 μmoles of R-1-P, 0.04 M Tris buffer, pH 7.6, 0.005 M $MgCl_2$, 0.01 M Versene, and 0.2 ml. of dialyzed extract of rabbit uterus (2.2 mg. of protein). Analyses were carried out as in Figs. 3 and 4. Curve C, the reaction mixtures contained 1.7 μmoles of R-1-P, 0.01 M succinate-Tris buffer, pH 7.3, 5×10^{-6} M GDP, 0.005 M $MgCl_2$, 0.005 M cysteine, and 0.1 ml. of a dialyzed extract of human uterus. Curve D, the reaction mixtures contained 2.3 μmoles of G-1-P, 0.01 M succinate-Tris buffer, pH 7.3, 5×10^{-6} M GDP, 0.005 M $MgCl_2$, 0.005 M cysteine, and 0.1 ml. of the dialyzed extract of human uterus. Analyses were carried out as in Figs. 3 and 4.

Rabbit Uterus—The whole uterus (wet weight 3 gm.) was first minced with scissors, and then ground in a mortar with 10 ml. of distilled water. The pulp was removed by centrifugation and the supernatant solution after a brief dialysis was tested in the usual enzyme assay. Phosphoglucomutase and phosphoribomutase activities are present in the extract of rabbit uterus (Fig. 5). Whereas in yeast the ratio of the activities was

approximately 100:1, in uterus the two activities were present in about equal amounts. As in the case of yeast, the presence of 0.01 M Versene had no effect on phosphoribomutase, while phosphoglucomutase was completely inhibited.

Bovine Uterus—Further evidence for the separate identities of these two enzymes was obtained during the course of purification of the enzyme of bovine uterus. Purification was carried out as follows. Uteri were obtained soon after removal from the carcass and were carried from the slaughter-house to the laboratory in a pail of ice. The organs could be stored at −15° without appreciable loss of activity.

Fat and other extraneous tissues were removed, the uterus was opened, and the endometrium scraped off and discarded. The remaining tissue, which is almost exclusively smooth muscle, was then processed; between 70 and 100 gm. were usually taken. This was first ground in a meat chopper, then homogenized for 2 to 5 minutes in a Waring blendor with 2 volumes of cold distilled water. The mixture was then allowed to stand for 15 minutes at 3°, centrifuged, and the precipitate discarded. The crude extract so obtained was dialyzed for 20 hours against 10 liters of distilled water; a precipitate which formed on dialysis was discarded. Approximately 10 ml. of this fraction (prolonged dialysis) were chilled, adjusted to pH 4 by the cautious addition of N acetic acid. No precipitation occurred, but when the pH was adjusted to 4.7 with N NaOH, the solution became turbid. After 15 minutes further chilling, the precipitate was removed by centrifugation and the supernatant solution adjusted to pH 6.5 (acid-supernatant fraction).

10 ml. of the acid-supernatant fraction were chilled in ice and adjusted to 0.60 saturation with ammonium sulfate by the slow addition of 15 ml. of a solution saturated at 25°, pH 8.1. After 15 minutes further chilling, the mixture was centrifuged and the precipitate discarded. The supernatant solution was adjusted to 0.80 saturation by the slow addition of 23.5 ml. of the ammonium sulfate solution. The turbid solution was chilled in ice for 15 minutes, and the precipitate collected by centrifugation, redissolved in 5 ml. of distilled water, and dialyzed overnight at 2° against distilled water (60 to 80 ammonium sulfate fraction). 2 ml. of a well aged calcium phosphate gel were added to 10 ml. of the 60 to 80 ammonium sulfate fraction. The mixture was stirred for 10 minutes, then centrifuged. Phosphoribomutase activity remained in the supernatant solution (Supernatant I). The final purification achieved by this procedure varied from 10- to 30-fold in several preparations.

As can be seen in Table II, there is a progressive decrease in the ratio of phosphoglucomutase activity to phosphoribomutase activity. A second fractionation with ammonium sulfate after the gel treatment usually resulted in further purification of phosphoribomutase, and in one of several

such preparations the final product was completely free of phosphoglucomutase. This change in ratio of the two activities can be ascribed only to the separation of two different proteins.

Like the phosphoribomutases of yeast and rabbit uterus, the enzyme from bovine uterus was active in the presence of 0.01 M Versene, while phosphoglucomutase activity was inhibited.

Human Uterus—A portion of tissue from a human uterus removed because of non-malignant disease was obtained, and an extract was prepared in the manner described above for bovine uterus. This extract also contained phosphoribomutase and phosphoglucomutase activity in approximately equal amounts (Fig. 5).

TABLE II

Separation of Phosphoribomutase and Phosphoglucomutase

Incubation mixtures for phosphoribomutase assay contained 1.2 μmoles of R-1-P, 10^{-6} M GDP, 0.02 M succinate-Tris buffer, pH 7.6, and 0.1 ml. of the appropriate enzyme fraction; final volume, 1.1 ml. Incubation mixtures for phosphoglucomutase assay contained 2 μmoles of G-1-P, 0.02 M succinate-Tris buffer, pH 7.6, 0.005 M $MgCl_2$, 10^{-6} M GDP, 0.005 M cysteine, and 0.1 ml. of the appropriate enzyme fraction; final volume, 1.1 ml. Reactions were stopped with perchloric acid, and the protein-free filtrates were analyzed for acid-labile phosphate. Unit activity is defined in the text.

Fraction	Unit of phosphoribomutase per mg. protein	Unit of phosphoglucomutase per mg. protein	Ratio of activities
Crude	0.032	0.039	1.22
Prolonged dialyzed	0.105	0.089	0.85
Acid supernatant	0.094	0.054	0.57
60–80 ammonium sulfate	0.340	0.050	0.15
Supernatant I	0.893	0.160	0.18

Human Blood—Blood was drawn by venipuncture, and 10 ml. were added to 2.0 ml. of 0.2 M Versene solution, pH 7.4. The blood was then centrifuged at room temperature and the serum removed by suction. The cells were washed into a dialysis tube with the aid of 3 ml. of distilled water and dialyzed for 24 hours at 2° against 1300 ml. of distilled water. The hemolysate so obtained was kept frozen. Both phosphoglucomutase and phosphoribomutase were present (Fig. 6). As in the other tissues tested, the phosphoribomutase reaction proceeded in the absence of added Mg^{++} and was active in the presence of 0.01 M Versene. In this case, however, there was some stimulation of the phosphoribomutase activity by cysteine and Mg^{++}. Whether this was due to stimulation of the magnesium-independent phosphoribomutase or to participation of phospho-

glucomutase or another enzyme present in the hemolysate remains uncertain.

Since the treatment to which the substrate and the various enzyme solutions were subjected would remove any loosely bound cations, if a divalent cation does take part in the reaction it must be bound so tightly to the protein that it is not available for complex formation with Versene. We have not yet investigated the phosphomutases of rabbit skeletal muscle, and Klenow gives no data for the ratios of activity toward R-1-P and G-1-P

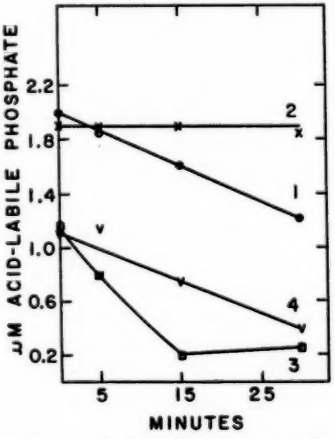


FIG. 6. Phosphoribomutase and phosphoglucomutase in hemolyzed blood. All incubated mixtures, final volume 1.20 ml., contained 0.20 ml. of the hemolysate, Tris buffer, 0.025 M, pH 7.5, and GDP, 1.6×10^{-6} M, with other additions as indicated. Curve 1, G-1-P, 1.6×10^{-3} M; cysteine, 7×10^{-3} M; $MgCl_2$, 4.2×10^{-3} M. Curve 2, G-1-P, 1.6×10^{-3} M; Versene, 1.2×10^{-2} M. Curve 3, R-1-P, 10^{-3} M; cysteine and $MgCl_2$ as above. Curve 4, R-1-P, 10^{-3} M; Versene, 1.2×10^{-2} M.

during the purification of yeast phosphoglucomutase; consequently it is possible that all the phosphoribomutase activity he found is indeed due to the phosphoglucomutases he studied.

SUMMARY

Evidence for the existence of a distinct phosphoribomutase has been presented. The conversion of R-1-P to an acid-stable phosphate ester has been demonstrated in extracts of bakers' yeast, brewers' yeast, rabbit, human, and bovine uterine muscle, and human blood. This enzyme has been shown to be different from phosphoglucomutase. The enzyme of bovine uterus has been partially purified and separated from phosphoglucomutase.

No demonstrable requirement for added divalent cations has been found, and the yeast enzyme is not inhibited by fluoride.

The pH optimum of the enzyme in yeast was found to lie between 6.7 and 7.2.

BIBLIOGRAPHY

1. Guarino, A. J., and Sable, H. Z., *Federation Proc.*, **13,** 222 (1954).
2. Sable, H. Z., and Calkins, C. W., *J. Biol. Chem.*, **204,** 695 (1953).
3. Klenow, H., *Arch. Biochem. and Biophys.*, **46,** 186 (1953).
4. Klenow, H., and Larsen, B., *Arch. Biochem. and Biophys.*, **37,** 488 (1952).
5. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124,** 397 (1938).
6. LePage, G. A., and Umbreit, W. W., *J. Biol. Chem.*, **148,** 255 (1943).
7. Posternak, T., *J. Biol. Chem.*, **180,** 1269 (1949).
8. Najjar, V. A., *J. Biol. Chem.*, **175,** 281 (1948).
9. Kalckar, H. M., *J. Biol. Chem.*, **167,** 477 (1947).
10. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162,** 421 (1946).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
12. Rowen, J. W., and Kornberg, A., *J. Biol. Chem.*, **193,** 497 (1951).
13. King, E. J., *Biochem. J.*, **26,** 292 (1932).
14. Mejbaum, W., *Z. physiol. Chem.*, **258,** 117 (1939).
15. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135,** 707 (1940).
16. Dickens, F., *Biochem. J.*, **32,** 1645 (1938).
17. Heppel, L. A., and Hilmoe, R. J., *J. Biol. Chem.*, **198,** 683 (1952).
18. Somogyi, M., *J. Biol. Chem.*, **160,** 69 (1945).
19. Meeraus, W., and Lorber, G., *Biochem. Z.*, **292,** 397 (1937).

MICROANALYSIS OF GLUCURONIDE GLUCURONIC ACID AS APPLIED TO β-GLUCURONIDASE AND GLUCURONIC ACID STUDIES*

By WILLIAM H. FISHMAN and SIDNEY GREEN

(From the Cancer Research and Cancer Control Unit, and the Department of Biochemistry and Nutrition, Tufts College Medical School, and the Cancer Research Laboratory, the New England Center Hospital, Boston, Massachusetts)

(Received for publication, December 28, 1954)

In recent years, interest in the metabolism of glucuronic acid has grown. Contributions have ranged from studies of its biosynthesis and conjugation in surviving tissues and experimental animals to its utilization in man (1–10). In all spheres, especially the latter, progress has been hampered by the lack of an analytical method by which glucuronides could be differentiated from free glucuronic acid in their mixtures.

The naphthoresorcinol reaction (11, 12) is widely used in metabolic studies, but is positive for both free and conjugated glucuronic acid. The principle of the proposed procedure depends on the selective oxidation of free glucuronic acid and other oxidizable interfering substances before the reaction with naphthoresorcinol. It has been possible also to improve the analytical performance of this latter reaction so that valid figures have been obtained for the small amounts of glucuronides present in human blood. Under special circumstances, both free and conjugated acid can be determined, making possible a number of important applications of the method, examples of which are presented.

Method

Principle—Glucuronic acid is eliminated from a mixture of the acid and glucuronides when the free aldehyde group is oxidized by hypoiodite at pH 10.1, forming saccharic acid which is insensitive to the naphthoresorcinol glucuronic acid reaction. Glucuronides are not affected by this treatment, since the aldehyde group is protected at carbon 1 by its linkage to the aglycone. Subsequently, the strongly acid conditions of the naphthoresorcinol test liberate glucuronic acid from its conjugate, giving rise to a violet-colored pigment, the amount of which is measured photocolorimetrically. An estimate of the unconjugated glucuronic acid is obtained

* Aided by a grant-in-aid from the Damon Runyon Memorial Fund for Cancer Research, by a fellowship from the Corn Products Refining Company, Argo, Illinois, and by an Institutional Grant of the American Cancer Society, New York. Presented before the 127th meeting of the American Chemical Society at Cincinnati, March 31, 1955.

by computing the difference in the values obtained for glucuronic acid before and after the oxidation procedure. This estimate is valid only in the absence of interfering amounts of hexoses.

Reagents—

Toluene (Merck reagent).

Ethyl alcohol, 95 per cent.

Buffer, pH 10.1. 8.4 gm. of sodium bicarbonate (Merck reagent) and 36.0 gm. of sodium carbonate (anhydrous, Merck reagent) are dissolved in a liter of distilled water.

0.1 N iodine in potassium iodide solution. It is stored (glass-stoppered amber-colored bottle) out of direct sunlight.

Sodium bisulfite solution (1.0 M). 10.4 gm. of sodium bisulfite (Merck reagent) are dissolved in 100 ml. of distilled water. A fresh solution is made up every 2 weeks.

6 N sulfuric acid. This solution may be prepared by diluting 1 volume of 18 N sulfuric acid with 2 volumes of water.

18 N sulfuric acid. 1 volume of concentrated sulfuric acid (Merck reagent) is poured into 1 volume of distilled H_2O and allowed to cool to room temperature before use.

Aqueous naphthoresorcinol solution, 0.4 per cent. A fresh solution is used for each day's determinations. It is essential to pulverize the naphthoresorcinol (Schwarz Laboratories, Inc., New York) with a mortar and pestle. It is necessary to shake the powdered naphthoresorcinol-water mixture in a glass-stoppered, amber-colored mixing cylinder for 10 minutes either by hand or with a rapid shaking apparatus. The filtered solution is protected from sunlight.

Apparatus—A Pyrex constant temperature bath, 16 inches in diameter, that includes a relay box, mercury thermocouple, stirrer, 500 watt immersion heater, and adjustable shelf manufactured by the Precision Scientific Company, Chicago, was used. Oil (Blandol, Howe and French, Inc., Boston) was substituted for water to maintain a constant immersion level of the tubes throughout the heating period. It was necessary to insulate the bath to avoid excessive heat loss and to install a 1000 watt auxiliary heater to shorten the time required to bring the temperature of the oil bath up to 100°. It is advisable to place the bath in a ventilating hood or underneath an exhaust vent.

Evelyn photoelectric colorimeter.

Procedure—5 ml. of solution are pipetted into an Erlenmeyer flask (50 ml. capacity) containing 2.05 ml. of carbonate buffer. 1.5 ml. of iodine solution are added, shaken gently, and the flask is stoppered and allowed to stand in the dark for 30 minutes. At the end of this time, 0.15 ml. of sodium bisulfite solution is added, the flask agitated, and an addition made

of 0.3 ml. of 6 N sulfuric acid. Any residual iodine coloration can be removed by 1 additional drop of bisulfite solution. The flask is shaken to remove the excess CO_2 from the solution. The subsequent determination of glucuronic acid by the naphthoresorcinol reaction on this mixture yields a value for glucuronide glucuronic acid.

To obtain a figure for total glucuronic acid, another 5 ml. of solution is pipetted into a solution containing iodine, bisulfite, and H_2SO_4 prepared in the amounts and in the sequence used before. This mixture is ready for the next step without the 30 minute interval.

Duplicate samples of 4 ml. aliquots are then pipetted into non-protein nitrogen type boiling tubes[1] with a ground glass mouth (purchased from the Macalaster Bicknell Company, Cambridge, Massachusetts). To each are added 2 ml. of 0.4 per cent naphthoresorcinol and 2 ml. of 18 N sulfuric acid (13). The contents of the tubes are mixed well, and the tubes are arranged, unstoppered, in a suitable metal rack and placed in the oil bath at 100° for 90 minutes. The oil level should be about 2 inches from the top of the tubes. The rack of tubes is then immersed in a container of cold water to which Alconox in the prescribed amount has been added. After cooling, 10 ml. of 95 per cent alcohol (14) are added to each tube, the tubes are shaken to dissolve the pigment, and 8 ml. of toluene (13) are added. Ground glass stoppers are inserted and the tubes vigorously shaken 100 times to extract the violet pigment into the toluene phase. The aqueous layer is removed completely by suction with a tube drawn out to a capillary attached to an aspirator and collecting bottle. The toluene extracts are then transferred carefully into Evelyn tubes.

All the above operations should be performed away from direct sunlight, since the pigment is sensitive to sunlight. After allowing the extracts to stand 5 minutes in the dark to permit them to clear, the optical density of each tube is measured in the Evelyn colorimeter at 565 mμ with the 6 ml. well, set to zero optical density with a toluene blank. Duplicate readings should agree within 2 galvanometer divisions. The reagent blank, which consists of 5 ml. of water, should not read below 85 per cent transmittance. It is also desirable to include a standard with each day's determinations.

Calibration Curve—5 ml. of solutions that contain 1.25, 2.5, 5, 10, and 16 γ of glucuronic acid[2] per ml., respectively, are pipetted into Erlenmeyer flasks that already contain the previously stated amounts of buffer, iodine, bisulfite, and acid (final volume, 9 ml.). *4 ml. aliquots of this mix-*

[1] The washing procedure for these tubes includes an alcohol rinse after use, then soaking in chromic acid solution. The tubes should be marked in lead pencil only on the ground glass spot.

[2] Glucuronolactone serves equally well as a standard, provided the data are corrected to glucuronic acid. Both pure glucuronic acid and glucuronolactone were generously provided by Dr. E. R. Weidlein and Dr. N. E. Artz.

ture, run in duplicate, are then pipetted for the naphthoresorcinol reaction. The concentration of glucuronic acid in the *4 ml. aliquot* is plotted against optical density to yield a straight line which is readily reproducible. A new curve should be run whenever a new batch of naphthoresorcinol or sulfuric acid is used.

Calculation—The average optical density of the duplicate readings is substituted in the standard calibration curve to yield the concentration of glucuronide glucuronic acid in 4 ml. The free glucuronic acid value is then obtained by subtracting the figure for glucuronide glucuronic acid from the total glucuronic acid value.

Solution of Glucuronides—

$$\gamma \text{ of glucuronide glucuronic acid} \times \frac{9}{4} = \gamma \text{ glucuronide glucuronic acid in 5 ml. analyzed}$$

Serum (1:10 Dilution in Protein-Free Filtrate)—

$$\frac{\gamma \text{ glucuronide glucuronic acid}}{1000} \times \frac{9}{4} \times \frac{10}{5} \times 100 = \text{mg. glucuronide glucuronic acid per 100 ml. serum}$$

Urine (Diluted 1:50)—

$$\frac{\gamma \text{ glucuronide glucuronic acid}}{1000} \times \frac{9}{4} \times \frac{50}{5} \times 100 = \text{mg. per 100 ml. urine}$$

EXPERIMENTAL

Measurement of Glucuronides and Glucuronic Acid in Aqueous Mixtures of Pure Compounds—Mixtures were made of glucuronides with glucuronic acid in varying proportions. The results of the analysis of 10 such mixtures are given in Table I. Agreement between expected and found amounts of glucuronides is reasonably good, even in mixtures favoring free glucuronic acid by a factor of 8 to 18. The standard percentage deviation from theoretical recovery of glucuronides in this series was ±3.3 per cent. The method is sensitive to glucuronic acid in a concentration of 0.50 γ per ml. or 2 γ in the test system. It has been our practice to work with concentrations of glucuronides or of glucuronic acid which yield values for optical density somewhere in the middle of the calibration curve.

Determination of Serum Glucuronides—2 ml. of serum are pipetted into 16 ml. of distilled water in an Erlenmeyer flask. The mixture is deproteinized by the addition of 1 ml. each of 10 per cent sodium tungstate and 0.66 N sulfuric acid, followed by filtration 10 minutes later. Two 5 ml. aliquots are taken for analysis as described above. Table II lists values for serum glucuronides obtained on healthy subjects and on ill patients.

The choice of serum rather than whole blood as the material for study represents a current trend, since it eliminates from the system glucuronic acid-rich leucocytes (15, 16).

The range of serum glucuronides is from 0.6 to 1.35 mg. per cent in healthy subjects, with occasional elevations in patients.

Previous workers (17) have introduced an empirical correction factor for blood glucose designed particularly for the study of diabetic serum glucuronic acid, which was considered to be abnormally high in most cases. However, the data obtained with the present method indicate clearly that serum glucuronides are within the normal range in diabetic patients. The non-glucuronide substances reacting in the naphthoresor-

TABLE I

Determination of Glucuronides and Glucuronic Acid in Mixtures of Known Composition

The results are in micrograms per ml.

Experiment No.	Total glucuronic acid		Free glucuronic acid		Glucuronide* glucuronic acid	
	Expected	Found	Expected	Found	Expected	Found
1	9.68	9.68	0.48	0.20	9.20	9.48
2	9.60	9.55	0.90	1.22	8.70	8.33
3	9.66	9.78	1.90	1.95	7.76	7.83
4	9.64	9.85	2.85	3.49	6.79	6.36
5	9.62	9.78	3.80	4.04	5.82	5.74
6	9.60	9.90	4.75	5.17	4.85	4.73
7	9.58	9.68	5.70	5.85	3.88	3.83
8	9.56	9.90	6.65	6.86	2.91	3.04
9	9.52	9.50	8.55	8.54	0.97	0.96
10	9.52	10.24	9.03	9.68	0.49	0.56

* The glucuronides of menthol, phenolphthalein, and pregnanediol in a ratio of 2:1:1 were dissolved in water to provide the stock solution of glucuronide, which was analyzed for glucuronide glucuronic acid. Mixtures of stock solution with a solution of glucuronic acid were prepared in the proportions stated.

cinol test in diabetic blood thus may be attributed to glucose. Studies on glucuronide formation in diabetic patients now become possible.

Determination of Urinary Glucuronides—Ordinary urines are diluted 1:50 with distilled water, and 5 ml. aliquots in duplicate are analyzed. Glucuronide-rich urines may require a 1:500 dilution. Some data on 24 hour urine collections on normal subjects appear in Table II.

Course of Glucuronide Formation in Humans—By utilizing the techniques described for measuring glucuronides, the study of the rate of conjugation of exogenous glucuronidogenic substances becomes possible. Fig. 1 illustrates the changes observed in the blood and urine of several normal subjects after the oral ingestion of menthol.

The advantages of the present analytical method over previous ones are

(1) that isolation of the urinary menthol glucuronide is not necessary before it can be determined and (2) that the small amounts present in blood serum can be measured. It is now possible to correlate rates of appearance

TABLE II

Values for Serum and Urinary Glucuronides

The results are in mg. per cent for serum glucuronides; in mg. per 24 hours for urinary glucuronides.

Subject No.	Serum glucuronides	Subject No.	Serum glucuronides
	Normal, healthy men		
1	1.01, 1.12, 0.85, 0.90	3	0.79, 0.79, 0.90, 0.79
2	0.79, 1.24, 0.73	4	1.00, 1.35, 0.62, 1.1
	Diabetic subjects		
1	1.7	8	1.2
2	1.2	9	1.2
3	1.9	10	1.0
4	1.4	11	1.0
5	1.1	12	1.2
6	1.3	13	1.0
7	1.2		
	Prostatic cancer patients		
1	1.6, 1.6, 0.9, 1.2	6	1.7, 1.6, 1.4
2	1.6, 1.4	7	1.2
3	1.4, 1.4, 1.4, 1.0	8	1.0
4	1.1, 0.8, 1.1	9	0.8
5	2.1, 6.8, 3.0, 0.9, 4.2		
	Normal, healthy men		
	Urinary glucuronides		
1	324, 356, 362		
2	400, 450, 387, 463		

of the glucuronide in blood and urine and to compute the percentage conversion of the substance administered to its glucuronide without its previous isolation. For menthol, this amounts to 62 to 66 per cent of the dose administered.

Assay of β-Glucuronidase Activity—Since, *in vitro*, β-glucuronidase catalyzes the hydrolysis of glucuronides, yielding the products glucuronic acid and the aglycone, the present techniques offer a means for assaying β-glu-

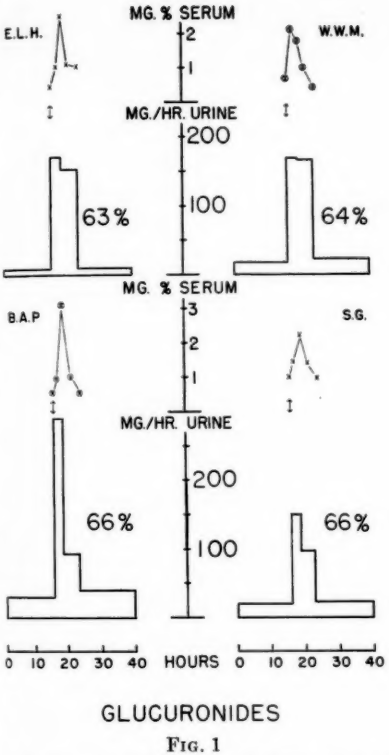


FIG. 1

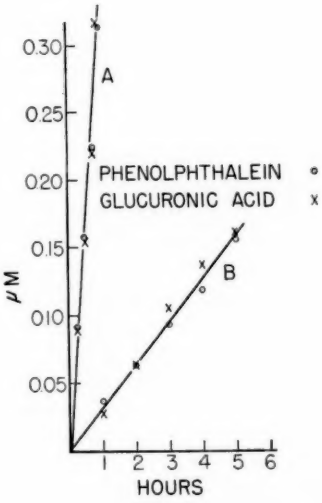


FIG. 2

FIG. 1. Alterations in blood serum and urinary glucuronides following the ingestion of menthol. All subjects except S. G. (1 gm.) received orally 1.5 gm. of menthol in aqueous suspension at 16 hours. Specimens of venous blood were taken at 16, 17, 19, 21, and 23 hours and the serum analyzed for glucuronide glucuronic acid. Urine collections were made at the intervals of 0 to 16, 16 to 19, 19 to 23, and 23 to 40 hours and were similarly analyzed. The conversion of administered menthol to the increment in urinary glucuronides has been computed in per cent.

FIG. 2. β-Glucuronidase-catalyzed hydrolysis of phenolphthalein glucuronic acid. The system contained 0.5 ml. of 0.01 M phenolphthalein glucuronic acid, 4 ml. of 0.1 M acetate buffer, pH 4.5, and 0.5 ml. of purified liver β-glucuronidase. In Curve A, the activity of the added enzyme solution was 500 units (21), and, in Curve B, 58 units per 0.5 ml. Such digests incubated at 38° in a water bath were removed at the intervals indicated and immersed in boiling water for 1 minute. Duplicate samples of 1 ml. each were taken for phenolphthalein measurement (21), and two 1 ml. aliquots were individually acidified with 0.1 ml. of 0.6 N H_2SO_4 solution, made up to 5 ml. with H_2O, and then extracted three times with 5 ml. of ethyl acetate. Analyses were performed on the aqueous phase for total and glucuronide glucuronic acid, from which data figures for free glucuronic acid were computed. The phenolphthalein and glucuronic acid liberated are expressed in micromoles per ml. of digest.

curonidase activity. Accordingly, a test was made of this idea by utilizing pure phenolphthalein glucuronide as the substrate and highly purified calf liver β-glucuronidase as the enzyme. The results are presented in Fig. 2.

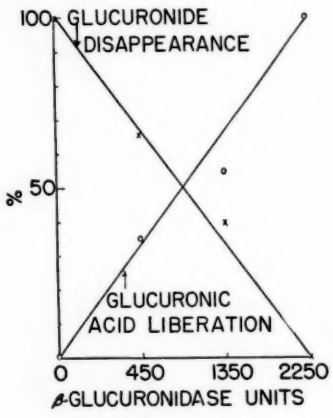


FIG. 3

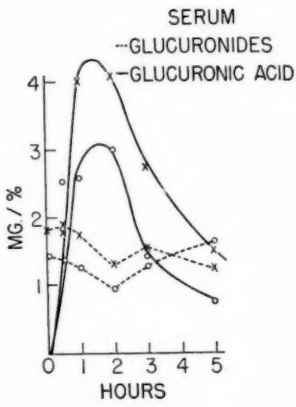


FIG. 4

FIG. 3. Appearance of free glucuronic acid and disappearance of glucuronide glucuronic acid upon incubating a new glucuronide with β-glucuronidase. The system contained 85 γ (glucuronide glucuronic acid) of an ethyl acetate-extractable product from a testosterone-liver slice incubation, in 3 ml. of aqueous solution plus 5 ml. of H_2O, 1 ml. of M acetate buffer (pH 4.5), and 1 ml. of purified liver β-glucuronidase. Three digests containing increasing amounts of enzyme as indicated in the figure were incubated for 4 hours at 37°. After deproteinization with 1 ml. of 10 per cent $ZnSO_4$ plus 2.5 ml. of 0.5 N NaOH and 0.5 ml. of H_2O, the supernatant fluids were analyzed for free and glucuronide glucuronic acid.

FIG. 4. Fluctuations of serum glucuronides and "glucuronic" acid in two human subjects observed after the ingestion of 5 gm. of glucuronolactone. The glucuronic acid figures were obtained by correcting the figures for oxidizable naphthoresorcinol-positive material in serum, expressed as glucuronic acid by the zero time value, it being assumed that the increment in serum was due to the glucuronolactone administered.

Measurements were made of the glucuronic acid liberated rather than of the disappearance of substrate so that these figures could be compared directly with values for phenolphthalein liberated. The good agreement found indicates that β-glucuronidase can be assayed by measuring glucuronic acid liberation by a more specific procedure than the determination of increase in reducing power employed previously. Moreover, phenolphthalein liberation has provided an independent yardstick with which to measure the performance of the present method for determining glucuronic

acid. It is expected that this assay will find application in the use of substrates whose aglycone moiety cannot be conveniently measured or when the enzyme acts on mixtures of glucuronides.

Identification of β-Glucuronides—β-Glucuronidase has been employed as an analytical agent to identify β-glucuronides. However, as a rule, no evidence for the liberation of free glucuronic acid is given, the identification of the aglycone alone being made after enzymic hydrolysis. On the other hand, instances may occur in which the amounts of material available are so small that identification of the aglycone by isolation and chemical characterization is not possible after β-glucuronidase hydrolysis. Thus, the use of present analytical techniques for establishing the presence of a new β-glucuronide formed after incubating testosterone with surviving rat liver slices[3] is illustrated in Fig. 3.

Utilization of Glucuronolactone by Human Subjects—It was not possible to distinguish between glucuronic acid and its conjugates by the methods used in previous studies. Information bearing on the question of the utilization of glucuronolactone in the formation of glucuronides is therefore lacking. Fig. 4 presents a demonstration of the fluctuations in serum glucuronides following the oral ingestion by humans of glucuronolactone. The increase in oxidizable naphthoresorcinol-positive material above the control level at zero time is ascribed to the blood level of the substance administered. These same conditions can be employed in studies of glucuronic acid utilization.

DISCUSSION

The use of alkaline iodine solutions for mild oxidation of aldoses was first described by Romÿn (18) in 1897 and has been widely used since (19). The conditions employed for glucuronic acid oxidation in this laboratory are essentially the same as those Levy and Doisy (20) used for quantitative oxidation of glucose. The oxidation procedure is efficient (see Table II), the conditions being adequate for the complete oxidation of at least 30 times the amount of glucuronic acid employed in these experiments. The product, saccharic acid, gives a negative naphthoresorcinol test. Glucuronides are completely resistant to this oxidation and can be measured successfully by the naphthoresorcinol color reaction after the oxidation step.

In order to obtain accurate data for the low levels of blood serum glucuronides, it was necessary to improve 2- to 3-fold the sensitivity and performance of the existing naphthoresorcinol reaction. This was accomplished by careful testing of conditions of heating, mineral acid and naphthoresorcinol concentration, and of the technique of pigment extraction.

[3] Fishman, W. H., and Sie, H.-G., *Federation Proc.*, **14**, 211 (1955).

XUM

Accuracy and reproducibility depended not only on good laboratory technique but also on the maintenance of even heating (oil bath at 100°) and on the close adherence to details of preparing the naphthoresorcinol solution. The standard curve was reproduced many times without exception, and duplicate transmission readings were as a rule within 1 per cent of each other in all the analyses.

The specificity of the procedure has also been studied. Whereas, with the use of the naphthoresorcinol reaction under conditions of lower acidity, glucuronic and galacturonic acids were the only materials which produced pigment to any important degree, it was found that hexoses and ascorbic acid registered with the present sensitive procedure. This interference is of no consequence in the determination of glucuronides, since it is eliminated by the oxidation step. However, this interference is important in interpreting the significance of naphthoresorcinol-positive oxidizable material in blood and urine. Thus far, no reports have appeared which identify free glucuronic acid as a component of normal blood. Our repeated attempts in this direction with the sensitive techniques of paper chromatography have been negative. Consequently, no significance with regard to glucuronic acid is now attached to this fraction in blood and urine except in cases in which glucuronic acid or the lactone is administered.

Confidence in the reliability and accuracy of the method is based both on satisfactory recovery of glucuronide glucuronic acid from mixtures with free acid and on the good agreement in the figures for phenolphthalein and glucuronic acid liberated in the enzymic hydrolysis of phenolphthalein glucuronic acid.

Examples of problems which can now be approached experimentally with the aid of the present method have been described to illustrate its potential applications and require no further discussion.

SUMMARY

1. Details are given of a procedure for determining glucuronides in the presence of glucuronic acid, which is based on the prior oxidation of the acid and other interfering aldehydic substances with alkaline hypoiodite solution, the remaining glucuronides being analyzed by the naphthoresorcinol color reaction.

2. Satisfactory recovery is obtained of glucuronides from their mixtures with glucuronic acid. The sensitivity of the method is 2 γ of glucuronide glucuronic acid and the standard deviation in the neighborhood of 3 per cent.

3. The procedure may be utilized for the assay of β-glucuronidase activity when the amount of the liberated aglycone is not readily determined.

4. The identification of β-glucuronides is made more certain by the new method, applied to β-glucuronidase hydrolysates.

5. The determination of the small amounts of glucuronides circulating in the blood is feasible, making possible time studies of the rates of conjugation and excretion of administered glucuronidogenic substances (*e.g.* menthol).

6. The course of absorption and excretion of administered glucuronolactone can be followed, and any alteration in the amount of excreted glucuronides can be easily measured.

BIBLIOGRAPHY

1. Mosbach, E. H., and King, C. G., *J. Biol. Chem.*, **185,** 491 (1950).
2. Storey, I. D. E., *Biochem. J.*, **47,** 212 (1950).
3. Bidder, T. G., *J. Am. Chem. Soc.*, **74,** 1616 (1952).
4. Packham, M. A., and Butler, G. C., *J. Biol. Chem.*, **194,** 349 (1952).
5. Doerschuk, A. P., *J. Biol. Chem.*, **195,** 855 (1952).
6. Dutton, G. J., and Storey, I. D. E., *Biochem. J.*, **57,** 275 (1954).
7. Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C., and Homburger, F., *J. Clin. Invest.*, **30,** 685 (1951).
8. Sudhof, H., *Z. physiol. Chem.*, **290,** 72 (1952).
9. Hollmann, S., and Wille, E., *Z. physiol. Chem.*, **290,** 91 (1952).
10. Fretwurst, F., and Ahlhelm, H. A., *Arch. exp. Path. u. Pharmakol.*, **217,** 382 (1953).
11. Tollens, B., and Rorive, B., *Ber. chem. Ges.*, **41,** 1783 (1908).
12. Mandel, J. A., and Neuberg, C., *Biochem. Z.*, **13,** 148 (1908).
13. Neuberg, C., and Kobel, M., *Biochem. Z.*, **243,** 435 (1931).
14. Maughan, G. B., Evelyn, K. A., and Browne, J. S. L., *J. Biol. Chem.*, **126,** 567 (1938).
15. Deichmann, W. B., and Dierker, M., *J. Biol. Chem.*, **163,** 753 (1946).
16. Follette, J. H., Valentine, W. N., Hardin, E. B., and Lawrence, J. S., *J. Lab. and Clin. Med.*, **43,** 134 (1954).
17. Saltzman, A., Caraway, W. T., and Beck, I. A., *Metabolism*, **3,** 11 (1954).
18. Romÿn, G., *Z. anal. Chem.*, **36,** 349 (1897).
19. Pigman, W. W., and Goep, R. M., Chemistry of the carbohydrates, New York (1948).
20. Levy, M., and Doisy, E. A., *J. Biol. Chem.*, **77,** 733 (1928).
21. Fishman, W. H., Springer, B., and Brunetti, R., *J. Biol. Chem.*, **173,** 449 (1948).

STUDIES ON THE SULFUR METABOLISM OF TISSUES CULTIVATED IN VITRO

I. A CRITICAL REQUIREMENT FOR L-CYSTINE

By JOSEPH F. MORGAN and HELEN J. MORTON

(From the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada)

(Received for publication, December 4, 1954)

The development of a chemically defined medium, Mixture 199, that supports the survival of mammalian tissues cultivated *in vitro* for approximately 35 days (1) has made it possible to study the nutrition and metabolism of tissue cells under precisely controlled conditions. During the development of this medium, it was observed by chance that omission of L-cystine from the complex mixture resulted in death of the experimental cultures within 8 to 12 days. Since L-cystine thus appeared to be a critical factor for cell survival, it was considered of interest to study the specificity of the cystine requirement, the rôle of sulfur-containing compounds in the nutrition of isolated tissue cells, and the metabolic pathways involved. The initial experiments, reported in the present communication, indicate that the sulfur requirements of isolated cells are very specific and differ markedly from those of the whole animal.

Materials and Methods

All cultures were prepared from the heart muscle of 11 day-old chick embryos. The tissue was reduced to a fine pulp by chopping with fine curved scissors, and aliquots of the resulting tissue suspension were transferred to standard Pyrex test-tubes by a capillary pipette. Cultures prepared in this way contained from 1 to 2 mg. of tissue, wet weight, as determined by frequent weighings of replicate samples. After a 25 to 30 minute period, during which the tissues became fixed to the glass, the synthetic medium was added, and the cultures were incubated at 37° by the conventional roller tube technique (2). Full details of the culture procedures have been reported previously (1, 3).

The synthetic medium employed throughout these experiments was designated Medium M 150 (4). It contains the 54 organic ingredients of Mixture 199 (1), but is prepared in a slightly different salt solution. These two media support cell survival for identical periods of time, but Medium M 150 was found preferable to Medium 199 because of its lower and more stable pH.

XUM

Media deficient in sulfur-containing amino acids were prepared by omitting from the formula for Medium 199 (1) L-cystine, DL-methionine, L-cysteine, and glutathione. For test purposes, the appropriate sulfur compounds were added to the deficient medium at final concentrations of 0.01, 0.1, 1.0, 10.0, and 100.0 mg. per liter. All compounds used were of the highest grade obtainable commercially and were employed without further purification. All media were prepared at frequent intervals and sterilized by passage through ultrafine fritted glass filters.

Each experiment consisted of 80 cultures, prepared at one time from a pooled lot of tissue. During the initial 7 day period (3), all cultures were grown in 0.75 ml. of Medium M 150. At the end of this time, the cultures were examined microscopically and divided into equivalent groups of ten to fifteen cultures. The control Medium M 150 was then removed and replaced in each tube by 1.5 ml. of experimental medium. The test media were renewed twice a week, and all cultures were examined microscopically three times a week until living cells could no longer be seen. The experimental results were then calculated as the average survival times of the groups of cultures in each test medium. The significance of differences in survival times was calculated by the standard t test.

Results

Response to Methionine, Cystine, and Methionine Plus Cystine—Preliminary experiments showed that omission of all sulfur-containing amino acids from the medium resulted in rapid death of the test cultures. The sheets of healthy spindle-shaped cells surrounding the original explants degenerated rapidly, and the individual cells became rounded and granular within a few days. When to this deficient medium were added graded levels of methionine alone, cystine alone, or graded levels of cystine in the presence of a constant amount of methionine, the curves shown in Fig. 1 were obtained.

It is apparent (Curve A) that L-methionine alone was ineffective in maintaining the survival of tissues cultivated in the deficient medium. A slight increase in survival was observed at a concentration of 10.0 mg. per liter, but statistical analysis of the data showed that this increase was barely significant at the 5 per cent level, and hence of doubtful value. Tests with D-methionine, which are not shown on the graph, gave no increase in survival at any concentration. When graded levels of L-cystine alone were incorporated, a definite response was obtained (Curve B). This response reached a maximum which corresponded to 66 per cent of the survival time normally observed with the complete medium, and then declined sharply. Repetition of the L-cystine experiment, in the presence of the level of DL-methionine ordinarily contained in Medium M 150 (30.0 mg. per liter), re-

sulted in a complete response (Curve C) which reached a maximum at 10.0 mg. per liter and then remained constant. The maximal values obtained in this curve are typical of the survival times normally obtained in the complete medium, No. M 150.

From these results, it would appear that the sulfur requirement of chick embryonic heart tissues cultivated *in vitro* cannot be satisfied by methionine alone, but can be partially satisfied by cystine alone. Only when *both* cystine and methionine are present, do the cultures survive for the full 35 day period normally obtained in Medium M 150. Since this response is

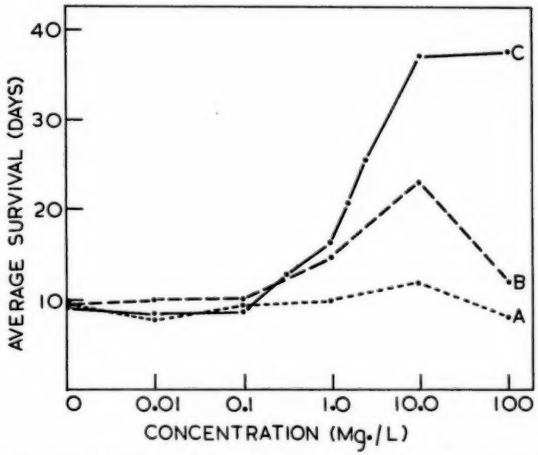


FIG. 1. Response of tissues cultivated in deficient media to additions of sulfur-containing amino acids. Curve A, graded levels of L-methionine alone; Curve B, graded levels of L-cystine alone; Curve C, graded levels of L-cystine in the presence of 30.0 mg. per liter of DL-methionine.

greater than the sum of the responses of cystine and methionine alone, a complex interrelationship between these amino acids is indicated.

Ability of Other Sulfur-Containing Compounds to Replace L-*Cystine*— Since previous experiments (Fig. 1) had shown that cystine, in the presence of methionine, was essential for cell survival, it was considered of interest to test the ability of other sulfur compounds to replace cystine in this biological system. In these tests, DL-methionine was included at 30.0 mg. per liter, since this concentration, in the presence of cystine, had been found to give the maximal response (Fig. 1, Curve C). The compounds studied were incorporated at final concentrations of 0.1, 1.0, 10.0, and 100.0 mg. per liter, since these levels corresponded to major points on the cystine response curve (Fig. 1, Curve C). The results of this survey are

recorded in Table I. Analysis of the data showed that increased survival times of less than 2 days were not significant, and, accordingly, such values have been recorded as zero in Table I.

TABLE I

Ability of Sulfur-Containing Compounds to Substitute for L-*Cystine*

Compound tested*	Increase in survival†	Optimal concentration	Level of significance, t test
	days	*mg. per l.*	
L-Cystine (control)	24.0	10.0	0.001
D-Cystine	0		
DL-Homocystine	0		
Dibenzoyl-L-cystine	0		
β,β'-Diaminoethyldisulfide	0		
DL-Homocysteine	0		
DL-Cystathionine	2.7	100.0	<0.05
L-Cysteine	20.0	10.0	0.001
D-Cysteine	0		
Glutathione (oxidized)	3.5	1.0	0.001
" (reduced)	0		
S-Acetyl glutathione	0		
Coenzyme A	0		
Thioctic acid‡	0		
Benzylpenicillin	0		
DL-Lanthionine	11.5	100.0	0.001
Djenkolic acid	0		
Thioglycolic acid	0		
2,3-Dimercaptopropanol§	2.6	0.1	0.001
6-Mercaptopurine‖	0		
Thiomalic acid	0		
Thiourea	0		
Ergothioneine	4.9	1.0	0.01
Thiolhistidine	0		
Sodium sulfate	0		
" thiosulfate	0		
" diethyldithiocarbamate	0		

* All compounds were incorporated at final concentrations of 0.1, 1.0, 10.0, and 100.0 mg. per liter in the test medium containing 30.0 mg. per liter of DL-methionine, but no cystine.

† Obtained by subtracting the average survival time of control cultures in the deficient medium from that of the experimental medium. Increases of 2.0 days or less were not found significant statistically and have been recorded as zero.

‡ Obtained through the courtesy of Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

§ Obtained through the courtesy of Mr. G. A. Grant, Defence Research Chemical Laboratories, Ottawa.

‖ Obtained through the courtesy of Dr. G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, New York.

It is evident (Table I) that L-cysteine almost completely replaced L-cystine, and that the effective concentrations of these two compounds (10.0 mg. per liter) were identical. A major degree of replacement was also observed with the highest level of DL-lanthionine tested, an observation that is in general agreement with the results of feeding experiments in rats (5). Minor degrees of replacement were obtained with ergothioneine, oxidized glutathione, and 2,3-dimercaptopropanol. Although the replacement values with these compounds were not high, statistical analysis of the data showed that the results were highly significant. In the case of DL-cystathionine, a response was obtained, but it was not significant.

Tissues cultivated in the sulfur-deficient medium consistently died within 8 to 12 days, as shown in Fig. 1. This uniform period of survival might be attributed to accumulation of intracellular reserve nutrients during the initial 7 days cultivation in the complete Medium M 150. It would appear difficult, therefore, under these cultural conditions, to cause death from nutritional deficiencies alone within a shorter period of time. When high concentrations of sodium diethyldithiocarbamate, 2,3-dimercaptopropanol, thiourea, or β,β'-diaminoethyldisulfide were added to the deficient medium, however, death of the cultures occurred within 3 to 7 days. This result would appear to indicate that these compounds exert a direct toxicity on the tissues over and above the sulfur deficiency already present, a toxicity that may possibly be related to the binding of heavy metals.

DISCUSSION

In the chick (6, 7) and in the rat (8, 9), L-methionine, but not L-cystine, is essential for growth. In the complete absence of methionine, the feeding of cystine fails to promote growth (10). The present experiments, which are based on more than 4000 tissue cultures, have shown that chick embryonic heart fibroblasts have a specific requirement for L-cystine, which cannot be satisfied by L-methionine. The presence of methionine, however, in addition to cystine, is required for optimal cell survival.

The observation that tissues will survive for only 8 to 12 days in media deficient in sulfur-containing amino acids, but will survive for 35 days if cystine and methionine are present, emphasizes the extreme importance of sulfur compounds to the cell. However, the present experiments have not yet indicated the specific function of these compounds. Since coenzyme A will not replace cystine, it appears unlikely that cystine is required for coenzyme A synthesis, as recently reported for *Lactobacillus arabinosus* (11).

The importance of cystine in the nutrition of tissue cultures had previously been recognized by Ehrensvärd, Fischer, and Stjernholm (12), employing partially synthetic media. These investigators observed that for proper cell growth in dialyzed plasma and embryo extract cystine was

necessary, but methionine was not. Under the present conditions, when completely synthetic media were used, cystine was found to yield a partial response, but for maximal cell survival the presence of methionine was also necessary. It is possible that, when non-synthetic media were used, the proteolytic activity of the tissue cultures may have released sufficient methionine from the proteins of the dialyzed plasma or embryo extract to satisfy the low requirement for this amino acid in the presence of added cystine.

The inability of methionine alone to satisfy the cell demand for sulfur-containing amino acids suggests that these tissues may be unable to demethylate methionine at a sufficient rate or that metabolic blocks may exist in the sequence of enzymic reactions leading from methionine to cysteine. The observation that cystathionine showed a measurable, but not statistically significant, response may indicate the possible location of the metabolic block. It is interesting to speculate whether the inability of the tissue to utilize methionine in the absence of cystine can be attributed to a lack of specific enzymes or coenzymes and on the possible rôle of vitamin B_{12} and choline in this biological system.

A characteristic feature of the present experiments has been the specificity of the cell requirement for each type of sulfur compound. Thus, although both L-cystine and L-cysteine were active, the D isomers were completely inactive. This is at some variance with earlier experiments which suggested that tissue cultures could slowly convert D-histidine into the L form (13). Similarly, oxidized glutathione exhibited a partial response, but no effect was obtained with reduced glutathione or *S*-acetyl glutathione. The partial replacement activity observed with ergothioneine, but not with thiolhistidine, is a further example of the specificity of the response.

The present studies have shown that chick embryonic heart fibroblasts have a critical requirement for sulfur-containing amino acids which differs markedly from the cystine-methionine relationship established by feeding experiments with chicks. Since a variety of animal tissues have been cultivated in synthetic media,[1] this method offers a new analytical tool for studying metabolic pathways in individual tissues freed of the complex interrelationships occurring in the whole animal.

SUMMARY

1. Chick embryonic heart fibroblasts cultivated *in vitro* in a completely synthetic medium remained alive for only 8 to 12 days in the absence of sulfur amino acids, but survived for approximately 35 days in the presence of these compounds.

[1] Morton, H. J., and Morgan, J. F., to be published.

2. Tissues cultivated in media deficient in sulfur amino acids responded to the addition of L-cystine alone, but not to L-methionine alone. Cell survival for the full 35 day period required the presence of L-methionine as well as L-cystine.

3. Of twenty-six sulfur compounds tested for ability to replace L-cystine in the presence of methionine, only L-cysteine showed nearly complete replacement. Minor degrees of replacement were observed with oxidized glutathione, lanthionine, ergothioneine, and 2,3-dimercaptopropanol.

The technical assistance of Miss M. A. McCrone, Miss D. Slessor, and Mr. L. F. Guerin is gratefully acknowledged.

BIBLIOGRAPHY

1. Morgan, J. F., Morton, H. J., and Parker, R. C., *Proc. Soc. Exp. Biol. and Med.*, **73,** 1 (1950).
2. Gey, G. O., *Am. J. Cancer*, **17,** 752 (1933).
3. Morton, H. J., Morgan, J. F., and Parker, R. C., *J. Cell. and Comp. Physiol.*, **38,** 389 (1951).
4. Burr, M. M., Campbell, M. E., Morgan, J. F., and Nagler, F. P., *Canad. J. Microbiol.*, **1,** 158 (1954).
5. Jones, D. B., Caldwell, A., and Horn, M. J., *J. Biol. Chem.*, **176,** 65 (1948).
6. Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, **138,** 467 (1941).
7. Grau, C. R., and Almquist, H. J., *J. Nutr.*, **26,** 631 (1943).
8. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **98,** 465 (1932).
9. White, A., and Beach, E. F., *J. Biol. Chem.*, **122,** 219 (1937–38).
10. Womack, M., Kemmerer, K. S., and Rose, W. C., *J. Biol. Chem.*, **121,** 403 (1937).
11. Pierpoint, W. S., and Hughes, D. E., *Biochem. J.*, **56,** 130 (1954).
12. Ehrensvärd, G., Fischer, A., and Stjernholm, R., *Acta physiol. Scand.*, **18,** 218 (1949).
13. Morgan, J. F., and Parker, R. C., *Arch. Biochem. and Biophys.*, **38,** 267 (1952).

METABOLIC FORMATION OF HOMOSERINE AND α-AMINOBUTYRIC ACID FROM METHIONINE*

By YOSHIHIKO MATSUO and DAVID M. GREENBERG

(From the Department of Physiological Chemistry, University of California School of Medicine, Berkeley, California)

(Received for publication, November 5, 1954)

Knowledge of the pathways of the metabolism of the carbon chain of methionine (1) is quite incomplete. Homoserine (2, 3) and α-aminobutyric acid (4) have been suggested on the basis of indirect evidence to be intermediates of methionine metabolism, but they have not hitherto been directly shown to be derived from this amino acid. The present communication provides evidence of this metabolic conversion.

Chromatographic separation of the metabolic products of methionine on a Dowex 50 column has been handicapped by the formation of methionine sulfoxide, which tends to overlap many other peaks. This has now been largely overcome by oxidizing the residual methionine to the sulfone.

EXPERIMENTAL

Chromatography on Dowex 50—The hydrochloric acid method of Stein and Moore (5) was employed. A resin bed of 0.9 × 110 cm. was prepared by packing the acid form of Dowex 50 into a 0.9 × 125 cm. Pyrex tube, the lower end of which was tightly plugged with glass wool. The eluting solutions employed were 130 ml. of H_2O, 200 ml. of 1.5 N HCl, and 200 ml. of 2.5 N HCl. The flow rate was 1 ml. in 15 minutes, and the fraction volumes were 1 ml. A requirement in this work was the removal of ammonia from the extracts to be chromatographed, as this substance tended to obscure the α-aminobutyric acid region of the chromatogram. The solution was accordingly adjusted to about pH 11 and placed over concentrated sulfuric acid overnight in an evacuated desiccator maintained at 37°.

Oxidation of Methionine to Sulfone—This step was found to be desirable in order to avoid the presence of methionine sulfoxide, possibly formed during the tissue extraction (4), which tends to spread over the chromatograms. Formation of this substance has been observed in this laboratory during the chromatographic procedure. Essentially according to the method of Toennies and Callan (6), the dried tissue extracts were dissolved in 1 ml. of a chilled oxidizing solution composed of 7 parts of 2.5 N HCl, 2

* Aided by research grants from the National Cancer Institute (No. C327 and C2327) and the Cancer Research Funds of the University of California.

XUM

parts of 30 per cent H_2O_2, and 1 part of 0.1 M sodium molybdate. After 1 hour at 0°, the solution was evaporated to dryness under reduced pressure over anhydrous phosphorus pentoxide and KOH pellets.

A test of this procedure was carried out by oxidizing a solution containing 10 mg. of DL-methionine containing 9×10^5 counts per 100 seconds and chromatographing the product on the Dowex 50 column. The distribution of the radioactivity is shown in Fig. 1. 98.9 per cent of the radioactivity was recovered, and 91 per cent of the activity appeared in Peak 4, the methionine sulfone peak. The substances in the minor peaks could not be identified, except that Peak 1 contained homocysteic acid.

Separation of Homoserine as Lactone—The presence of glycine and alanine in the homoserine peak made it imperative to isolate the latter substance in order to establish the conversion of methionine-2-C^{14} to homoserine. This was accomplished by converting homoserine to the lactone with concentrated HCl and chromatographing the lactone through a short column of the ammonium form of Dowex 50 with an acidic ammonium formate buffer as the eluting solvent.

The fraction from the HCl-Dowex 50 chromatogram was dissolved in a few ml. of concentrated HCl and refluxed for 1 hour at 100°. The HCl was then removed by evaporation, and the dried material was dissolved in a small volume of the eluting buffer. This solution was transferred to a 0.6×7 cm. resin bed which was packed with the ammonium Dowex 50 saturated with the eluting solution. The latter consisted of 0.2 M ammonium formate at pH 3.1, containing 40 per cent by volume of ethanol. (The pH was measured before the addition of the ethanol.) The flow rate was 0.1 to 0.2 ml. per minute, and the fraction volumes were 5 ml.

The lactone is bound more firmly than the contaminating amino acids. The separation obtained with this procedure on a test mixture is shown in Fig. 2 for 10 mg. quantities of each of the three amino acids under consideration. Homoserine lactone was eluted in a discrete peak (B), and alanine and glycine were eluted in a single peak (A). Because of the small resin bed and the relatively large fraction volumes, no complete separation is achieved among the individual neutral and acidic amino acids.

The completeness of the separation is indicated by the fact that homoserine, isolated from a mixture containing radioactive alanine and glycine, was free of radioactivity. The same result was obtained with a mixture of homoserine and labeled phenylalanine. Since phenylalanine is the last of the neutral and acidic acids to emerge from the column under our conditions, homoserine isolated by this technique should be free from any of the amino acids of this group.

It was also demonstrated that the basic amino acids are eluted after the homoserine lactone.

Animal Experiments—Male mice about 20 gm. in body weight were injected intraperitoneally with 0.4 ml. of a solution containing 2.5 mg. of DL-methionine-2-C^{14} (5.6×10^6 counts per 100 seconds), and 5 mg. each of DL-homoserine, DL-α-aminobutyric acid, and DL-serine. The homoserine

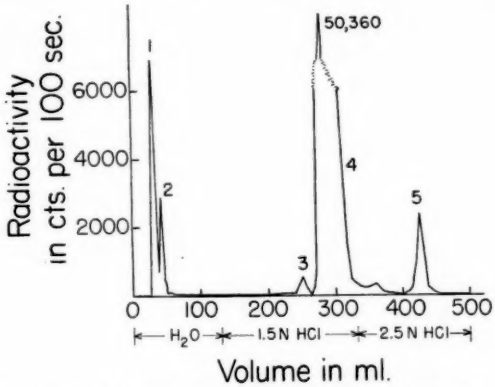


FIG. 1. Chromatogram of products of oxidation of methionine to methionine sulfone. Distribution of radioactivity in per cent: Peak 1, 3.4; Peak 2, 1.8; Peak 3, 0.8; Peak 4, 91.1; Peak 5, 2.9.

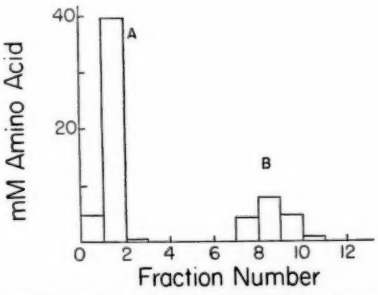


FIG. 2. Chromatographic separation of homoserine lactone from glycine and alanine. Peak A, alanine and glycine; Peak B, homoserine lactone.

and α-aminobutyric acids were employed to trap the radioactive compounds and the serine to promote the formation of cystathionine, which is conceivably on the pathway of the formation of homoserine from methionine.

The mice were killed 1 hour after the injection, the skin and tail were removed, and the carcass was steamed in an autoclave at 105° to soften the tissues. The bones were carefully removed, and the soft tissues were homogenized in a Potter-Elvehjem all-glass homogenizer in an equal vol-

ume of 5 per cent perchloric acid. The homogenate was centrifuged, and the supernatant fluid was freed from as much as possible of the floating fat and decanted. The precipitate was extracted three times with 30 ml. portions of the acid, and the combined perchloric acid extracts were neutralized with 2 M KOH solution to pH 7 to 7.5. The whole operation required about 40 minutes, and the temperature of the preparation was maintained at about 5° to minimize the possible destructive action of perchloric acid. The precipitate formed after neutralization with KOH was removed, and the slightly alkaline supernatant fluid was evaporated on a steam bath to about 5 ml. Any precipitate that formed was removed by centrifugation, and the supernatant fluid was evaporated to dryness in *vacuo*.

Radioactive Material and Measurement—The radioactive methionine was purchased from Tracerlab, Inc., Boston. The fractions from the chromatography columns were collected and dried (with gentle heat) in polyethylene planchets and then were counted for 100 seconds in a gas flow counter. Fractions with counts above 300 per second were counted with a thin mica end window Geiger-Müller counter. These values were converted to gas flow counter counts by multiplying by the counting ratio between the two tubes, determined with a C^{14} standard sample.

Results

In a separate experiment it was determined that, in the period of these experiments, about 20 per cent of the label of the DL-methionine-2-C^{14} was eliminated as respiratory CO_2.

A typical chromatogram obtained in these experiments (five chromatograms in all) is presented in Fig. 3 and the distribution of the radioactivity among the different peaks recorded in Table I.

Peaks A, B, F, and K are identical with Peaks 1, 2, 3, and 5 of Fig. 1, which are formed non-enzymatically upon oxidation of the methionine.

Methionine Sulfone Peaks (G and H)—The partially overlapping peaks, G and H, appeared in all experiments. They emerge from the column just after the non-radioactive peak of glutamic acid. Peak G appears in the region expected for methionine sulfone, which gives only one peak when chromatographed alone (Fig. 1). Paper chromatograms run in phenol-water (80:20), acetic acid-butanol-water (2:3:1), and *tert*-amyl alcohol-pyridine-water (35:35:25) showed the presence of methionine sulfone and glutamic acid as the main components of both Peaks G and H (Fig. 4). Only the methionine sulfone was radioactive.

A reasonable explanation of two peaks is that Peak G represents mainly free methionine sulfone and Peak H is composed of a conjugate of methionine sulfone and glutamic acid.[1] Such a conjugate may well be hydrolyzed

[1] Excretion of such a conjugate into urine upon feeding methionine sulfone to the rat has been reported (7).

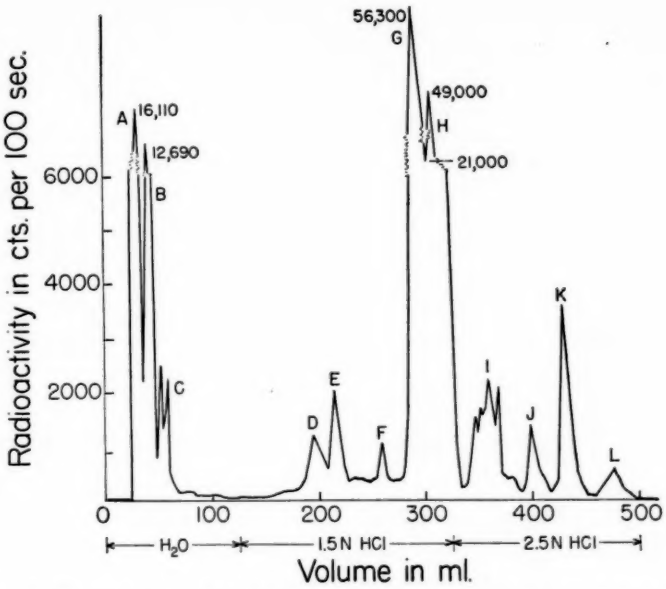


FIG. 3. Chromatogram of perchloric acid extract of tissues of mouse injected with DL-methionine-2-C^{14}.

TABLE I

Distribution of Radioactivity in Chromatogram of Metabolic Products of Methionine

Peak	Activity, counts per 100 sec. $\times 10^{-4}$	Per cent of total recovery	Chemical nature
A	9.6	7.2	Contains homocysteic acid
B	5.3	4.0	Unknown
C	1.9	1.4	"
D	1.3	1.0	"
E	2.3	1.7	"
F	0.7	0.5	"
G	42.4	31.8	Methionine sulfone
H	59.4	44.6	" " conjugate
I	4.4*	3.3	Contains homoserine
J	1.5	1.1	α-Aminobutyric acid
K	3.7	2.8	Unknown
L	0.7	0.5	"

* 1.6×10^4 counts per 100 seconds due to homoserine.

when dried in hydrochloric acid solution on the planchets, thus revealing only its components upon paper chromatography. Evidence in support of this is as follows: (1) The only radioactive material in both peaks is methionine sulfone, and it alone yields only one peak. (2) Upon chromatographing different regions of Peaks G to H, it was found that the glutamic acid spots decreased in intensity through the region of Peak G, with no relation to the rise and fall of the intensity of the methionine sulfone spots. This finding indicates that the glutamic acid in the region of Peak G is derived from the trailing of the glutamic acid which precedes the methionine sulfone. In the region of Peak H, the rise and fall in the intensity of the glutamic acid spots parallels that of the methionine sulfone. (3) When the fractions of peaks G and H were recombined and rechromatographed

FIG. 4. Drawing of a photograph of paper chromatograms of Peaks G and H of Fig. 3, showing the presence of glutamic acid as evidence for the presence of a methionine sulfone-glutamic acid conjugate. Solvent, *tert*-amyl alcohol-pyridine-water (35:35:25). The fractions of Peak G (293) and Peak H (308) were run with a known sample of glutamic acid (Glu) and methionine sulfone (MSO_2).

on an HCl-Dowex 50 column, the radioactive methionine sulfone appeared only in Peak G. A non-radioactive peak of glutamic acid appeared before Peak G. This shows that once the conjugate has been decomposed, liberating glutamic acid and methionine sulfone, recombination does not occur.

Identification of Homoserine in Peak I—In the mice experiments, Peak I was rechromatographed to isolate the lactone. Of 4.4×10^4 counts in the peak, 1.6×10^4 counts were isolated as homoserine, and 2.5×10^4 counts were eluted in the glycine-alanine peak. No attempts were made to identify the compound (or compounds) responsible for the radioactivity of this peak.

To establish further the identity of the radioactive homoserine it was chromatographed on paper. The isolated lactone was treated with warm 0.1 M NaOH solution, carefully neutralized to pH 7.5 with HCl, and most of the resulting NaCl removed by adding ethanol to a concentration of about 90 per cent. In the paper chromatograms the radioactivity was contained in the homoserine spot. There were also a negligible spot of

homoserine lactone and a faint trace of another ninhydrin-positive spot in which the radioactivity was below the limit of detection.

To the remainder of the radioactive homoserine there were added 80 mg. of inert DL-homoserine, and the mixture was recrystallized a number of times from an acetone-water mixture. The specific activity remained constant as shown by the following: third recrystallization 42.3 ± 0.4[2] c.p.m. per mg.; sixth recrystallization 41.6 ± 0.6 c.p.m. per mg.

Evidence for Radioactive α-Aminobutyric Acid in Peak J—The evidence for this is as follows: (1) Its position on the column chromatogram coincides with that of authentic α-aminobutyric acid. (2) The ninhydrin curve coincided with the radioactivity curve. (3) Paper chromatograms run with three different solvents showed that the ninhydrin-positive substance and the radioactivity coincided with α-aminobutyric acid. (4) Addition of carrier α-aminobutyric acid to the isolated radioactive material and recrystallization from 95 per cent ethanol yielded the following constant specific activities: fourth recrystallization, 9.4 ± 0.3 c.p.m. per mg.; sixth recrystallization, 10.1 ± 0.7 c.p.m. per mg.

There are reports in the literature (8) that under certain conditions α-aminobutyric acid can be formed non-enzymatically from threonine. This has no significance in the present work, since no radioactivity was present in the threonine fractions.

DISCUSSION

The experimental results provide evidence for the conversion of the carbon chain of methionine to homoserine and α-aminobutyric acid. The per cent conversion of administered methionine to these products is low (Table I). The presence of a large amount of the products of metabolically unchanged methionine is responsible to some extent for the apparently low yields of these metabolic products. In addition homoserine was found to be rapidly metabolized in yeast cells (9). An enzyme system has been demonstrated in rat liver which converts homoserine to α-ketobutyric acid (2, 10, 11), and its activity is reported to be high (11). α-Aminobutyric acid also is known to be readily metabolized by animal tissues (12).[3]

The present experiments do not provide an insight into the exact metabolic pathways of the formation of homoserine and α-aminobutyric acid. The use of more immediate precursors and isolated enzyme systems may be required to secure such information.

Credit for the initial experimental work on this problem is due to Dr.

[2] Standard deviation.

[3] Also from unpublished data from this laboratory.

Mansoor ul Hassan, who obtained evidence for the formation of radioactive α-aminobutyric acid. His departure for the School of Medicine, Johns Hopkins University, prevented him from continuing the work.

SUMMARY

The metabolic formation of homoserine and α-aminobutyric acid from methionine has been demonstrated. This was accomplished by injecting DL-methionine-2-C^{14} into mice and isolating the radioactive compounds by column chromatography and characterizing them by paper chromatography and by the demonstration of constant specific activity on recrystallization with carrier.

Methods have been developed for the column chromatography on Dowex 50 of methionine derivatives by oxidizing methionine to the sulfone. Isolation of homoserine is accomplished by converting it to the lactone and subsequent chromatography on a short column of ammonium Dowex 50.

BIBLIOGRAPHY

1. Greenberg, D. M., Chemical pathways of metabolism, New York, **2,** 73 (1954).
2. Carroll, W. R., Stacy, G. W., and du Vigneaud, V., *J. Biol. Chem.*, **180,** 375 (1949).
3. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172,** 651 (1948).
4. Dent, C. E., *Science*, **105,** 335 (1947).
5. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 179 (1949).
6. Toennies, G., and Callan, T. P., *J. Biol. Chem.*, **129,** 481 (1939).
7. Wingo, W. J., Smith, R. A., and Wood, J., *Arch. Biochem. and Biophys.*, **47,** 307 (1953).
8. Heyns, K., and Walther, W., *Z. physiol. Chem.*, **294,** 111 (1954).
9. Schlenk, F., and Smith, R. L., *J. Biol. Chem.*, **204,** 27 (1953).
10. Binkley, F., and Olson, C. K., *J. Biol. Chem.*, **185,** 881 (1950).
11. Braunstein, A. E., and Azarkh, R. M., *Doklady Akad. Nauk S. S. S. R.*, **85,** 385 (1952); *Chem. Abstr.*, **46,** 11,267 (1952).
12. Cammarata, P. S., and Cohen, P. P., *J. Biol. Chem.*, **187,** 439 (1950).

PHOSPHORYLATION COUPLED TO THE OXIDATION OF FERROCYTOCHROME *c**

By SIGURD OLAF NIELSEN† and ALBERT L. LEHNINGER

(From the Department of Physiological Chemistry, School of Medicine, The Johns Hopkins University, Baltimore, Maryland)

(Received for publication, November 12, 1954)

The oxidation of ascorbic acid to dehydroascorbic acid by suspensions of mitochondria supplemented with cytochrome *c*, adenosine diphosphate (ADP), orthophosphate, and Mg^{++} causes a coupled aerobic phosphorylation with measured P:O ratios (moles of P uptake per atom of oxygen uptake) approaching the value 1.0 (2, 3). It has been concluded tentatively that the phosphorylation observed is actually coupled to electron transport between ferrocytochrome *c* and oxygen, since ascorbic acid apparently acts as a non-enzymatic reductant of ferricytochrome *c* in such experiments. However, this type of experiment is not completely conclusive because of the possible occurrence of oxidative or phosphorylative events uniquely associated with the presence of ascorbic acid (4, 5).

In this paper are recorded direct measurements of phosphorylation coupled to the oxidation of purified ferrocytochrome *c*, added to the mitochondrial test system as substrate. The presence of non-enzymatic reductants of cytochrome *c* in the test medium was therefore completely avoided. The P:O ratios observed were found to be in general agreement with those earlier observed with ascorbic acid as reductant. The data therefore establish directly that one of the three phosphorylations known to be coupled to the respiratory chain between reduced diphosphopyridine nucleotide and oxygen (6) occurs during electron transport between ferrocytochrome *c* and oxygen.

EXPERIMENTAL

Preparative—Ferro- and ferricytochrome *c* were prepared from horse heart cytochrome *c* as purchased from the Sigma Chemical Company, or following purification by the method of Keilin and Hartree (7). To prepare ferrocytochrome *c*, 5.0 ml. of neutralized 4×10^{-4} M cytochrome *c* and about 0.15 gm. of thoroughly washed palladium-asbestos catalyst (5 per

* Supported in part by grants from the National Institutes of Health and the Nutrition Foundation, Inc. A preliminary report of this investigation has been published (1).

† Fellow of the Egmont H. Petersen Foundation, Copenhagen, and Fulbright Travel Fellow.

cent, Fisher) were shaken gently at room temperature in a flask, first for 15 minutes under an atmosphere of nitrogen, then for 30 to 45 minutes under an atmosphere of hydrogen, and finally under nitrogen again. The catalyst was removed by filtration and the pH readjusted to 7.4. The reduction was usually 95 to 100 per cent complete with Sigma cytochrome *c* and somewhat less with the purified cytochrome *c*, as determined spectrophotometrically. Solutions of ferricytochrome *c* were prepared by adjusting a 4×10^{-4} M solution to pH 2.5 with 1 N HCl and exposing the solution to air in a thin layer with occasional shaking for 30 to 45 minutes. The pH was readjusted to 7.4 with 1 N NaOH. The oxidation was usually 98 to 100 per cent complete. The stock solutions of ferro- and ferricytochrome *c* were kept frozen at −15°.

$H_3P^{32}O_4$ was obtained from the Oak Ridge National Laboratory. It was heated 1 hour at 100° in 0.35 N HCl (to convert metaphosphoric and pyrophosphoric acids to orthophosphoric acid) before dilution with 0.005 M KH_2PO_4 to a specific activity of about 1.5×10^6 c.p.m. per μatom of P. Under the counting conditions used, 1 mc. of P^{32} corresponded to about 6×10^7 c.p.m. The pH was brought to 7.4 with 1 N NaOH. Such stock solutions were kept frozen at −15°. Less than 0.1 per cent of the radioactivity could not be removed by repeated extractions with benzene-isobutanol mixtures following additions of carrier phosphate and acid molybdate (8); this corresponds with delivery specifications which indicate a radiochemical purity of at least 99.9 per cent. Counting of radioactivity was carried out to 2 per cent precision.

Pure sodium fluoride free of divalent metal ions was prepared by slowly adding saturated NaCl solution to a filtered, neutral, saturated solution of $KF \cdot 2H_2O$ (Mallinckrodt, analytical) containing 0.07 M sodium ethylenediaminetetraacetate (Versene). The precipitate formed by the first drops of NaCl was discarded; the bulk of the precipitated sodium fluoride was then collected and washed three times with cold water before drying at room temperature.

Rat liver mitochondria, washed three times with 0.25 M sucrose, were prepared exactly as described before (2) and made up to a stock suspension in 0.25 M sucrose, so that 1.0 ml. contained the mitochondria derived from 1.0 gm. of fresh rat liver. The "working" suspension was made up between 10 and 15 minutes prior to the phosphorylation test by adding 0.025 ml. of the stock suspension to 10 ml. of ice-cold 0.075 M sucrose + 0.001 M Versene, pH 7.4, and mixing. After 5 minutes, 0.1 ml. of 50 per cent ethanol or acetone containing 1.0 γ of antimycin A (obtained through the courtesy of Professor Frank M. Strong, University of Wisconsin) was added to the "working" suspension of mitochondria and mixed. Aliquots of this suspension were added to the reaction cuvettes as described below.

Methods—The general plan of the P:O determinations was as follows. The rate of oxidation of small amounts of ferrocytochrome *c* at the expense of dissolved oxygen was followed spectrophotometrically in a cuvette containing mitochondria, ferrocytochrome *c*, orthophosphate labeled with P^{32}, and adenosine diphosphate as phosphate acceptor. At required times the reaction was stopped by addition of trichloroacetic acid (TCA), and the newly formed "esterified" P^{32} was measured by the procedures outlined below. Since earlier work had established (2) that maximal P:O ratios for the oxidation of ferrocytochrome *c* could be observed only at concentrations of the latter no higher than about 5×10^{-5} M, it was necessary to employ methods capable of measuring the uptake, in a 1.0 ml. reaction medium, of a few millimicromoles of orthophosphate (P_i) from a very much larger pool of orthophosphate, accompanying the oxidation of a few millimicromoles of ferrocytochrome *c*. Details of the procedures employed follow.

The reactions were carried out in cuvettes of 1.0 cm. optical path. These were loaded at room temperature from a previously prepared mixture of radiophosphate, $MgCl_2$, ADP, tris(hydroxymethyl)aminomethane buffer (Tris), Versene, and water, which was adjusted to pH 7.4 (glass electrode). An aliquot of this mixture was frozen and held for analysis of orthophosphate and P^{32} content, from which the specific activity of the orthophosphate was calculated. The remaining additions were cytochrome *c* and mitochondria, neither of which contributed significant amounts of orthophosphate to the reaction medium. Final concentrations of all components are given in Table I. Cytochrome *c* and the other reaction components were then added to make the volume in the test cuvettes 0.80 ml. (exclusive of mitochondria). The optical density at 550 mμ (absorption maximum for ferrocytochrome *c*) was determined against a water blank in a Beckman DU spectrophotometer equipped with a photomultiplier and a "pinhole" diaphragm between the light slit and the cuvette. The slit was set at 0.01 mm., corresponding to a band width of 1.4 mμ at 550 mμ. Following the reading at 550 mμ, a second reading was made at 557 mμ (an isosbestic point in the absorption spectra of ferro- and ferricytochrome *c*). At time zero, 0.20 ml. of the "working" mitochondrial suspension in 0.075 M sucrose containing Versene and antimycin (equivalent to the mitochondria derived from 0.5 mg. of fresh liver) was added, and the contents of the cuvette mixed to make a total volume of 1.0 ml. A second reading was taken at 557 mμ; the timing of the second is not critical, since the extinction coefficients of ferro- and ferricytochrome *c* are equal at this wave-length.

The wave-length adjustment was then quickly returned to 550 mμ and timed readings of optical density recorded. The reaction was stopped by the timed addition to each cuvette of 0.4 ml. of 25 per cent TCA made 0.05

M with respect to KH_2PO_4. The optical density at the exact time of fixing was accurately determined by extrapolation from the reaction curve which was plotted for each cuvette. Total reaction times as short as 2.0 minutes could be studied; most experiments involved periods of 10 to 15 minutes. The fixed reaction medium was analyzed for phosphate uptake as described below.

The molecular oxygen dissolved in the air-equilibrated reaction medium (approximately 0.5 μatom per ml.) represented a 50- to 100-fold excess over that actually consumed in the oxidation of the added ferrocytochrome during the course of the phosphorylation test.

As many as seven reaction cuvettes could be handled simultaneously by one operator. In addition a like number of reactions in which the optical density changes were either of little interest or known to be negligible could be carried out in test-tubes. One cuvette was frequently used to check the usually negligible rate of autoxidation of ferrocytochrome *c* in the absence of added mitochondria. No attempt was made to maintain rigorous temperature control since the P:O measurements are essentially independent of small temperature changes. The reactions were carried out at approximately 23–26°.

The spectrophotometric readings at 557 and 550 mμ described made possible the calculation of the exact change in optical density from the time of addition of mitochondria to the completion of the reaction, even if readings at 550 mμ were not started until several minutes after the addition of mitochondria, as was usually the case when several vessels were run simultaneously. The optical density of any given suspension of mitochondria at 557 mμ was found to be equal to that at 550 mμ under the conditions described (about 0.020 to 0.030 for different preparations). This value was added to the optical density of the ferrocytochrome *c* which was measured at 550 mμ *before* addition of mitochondria, multiplied by the dilution factor 0.8. The sum is the optical density of the complete system at 550 mμ at time zero. The observed decreases in optical density at 550 mμ as the ferrocytochrome *c* was oxidized were then used directly to calculate the amount oxidized, since the optical density contribution of the mitochondria was found not to change significantly over the period of the test.

The P:O ratio (moles of P uptake per atom of oxygen used) requires 2 electrons for the reduction of 1 atom of oxygen. Since each molecule of ferrocytochrome *c* oxidized provides but 1 electron for reduction of oxygen, the P:O ratio used in this paper is defined as follows:

$$\text{(1)} \qquad \text{P:O} = \frac{\text{mμmoles orthophosphate taken up}}{\text{0.5 mμmole ferrocytochrome } c \text{ oxidized}}$$

The expression for calculating the denominator of Equation 1 from the

experimental data is then

$$(2) \quad -\tfrac{1}{2}\Delta Fe^{II} \text{ cytochrome } c = \frac{0.8(D_{550} - D_{557}) - (d_{550} - d_{557})}{2(28.3 - 8.8)} \times 1000 \text{ m}\mu\text{moles}$$

where D and d, respectively, are the optical densities before and after the addition of mitochondria, at the wave-length of the subscripts. d_{550} is the optical density extrapolated to the exact time that the reaction is stopped. The molar extinction coefficients of Theorell and Åkesson (9) were used; for ferrocytochrome c, $K = 28.3 \times 10^6$ sq. cm. per mole and, for ferricytochrome c, $K = 8.8 \times 10^6$ sq. cm. per mole ($\log_{10}$), both at 550 mμ.

For determination of orthophosphate uptake, the following procedure, a modification of the method of Ernster *et al.* (10), was employed. After addition of 0.4 ml. of TCA-KH_2PO_4 to the 1.0 ml. of reaction medium, a 0.60 ml. aliquot of the slightly turbid mixture, which showed very little tendency to settle out, was added to a glass-stoppered centrifuge tube containing 1.2 ml. of acetone and mixed. 10 minutes later, 1.90 ml. of water (saturated with isobutanol) and 7.0 ml. of a water-saturated mixture of equal volumes of benzene and isobutanol were added, and the mixture was thoroughly shaken. After separation of the two phases, 0.80 ml. of the acid molybdate reagent of Martin and Doty (8) was added to the aqueous layer, gently mixed with it, and the tube allowed to stand 5 minutes. The tube was then shaken vigorously for 30 seconds; this operation extracts phosphomolybdic acid into the isobutanol-benzene layer. The aqueous (lower) layer was then removed with a capillary pipette (quantitative removal unnecessary) and filtered through Whatman No. 50 paper. The filtrate was collected in a second glass-stoppered centrifuge tube. After adding 0.03 ml. (1 drop) of 0.02 M KH_2PO_4 (carrier) and 3.5 ml. of water-saturated isobutanol-benzene, the contents were vigorously shaken for 30 seconds. Following these two extractions with isobutanol-benzene, the aqueous layer was withdrawn by capillary pipette and exactly 1.0 ml. counted "wet" in an aluminum cup under constant geometry. From the specific activity (A) of the orthophosphate present in the reaction medium (counts per minute per millimicromole of orthophosphate), the radioactivity of the 1.0 ml. aliquot of "esterified" P separated as above (B) (counts per minute), and appropriate factors for aliquot size, dilution, etc., the amount of orthophosphate uptake per 1.0 ml. of the original reaction medium, can be calculated:

$$(3) \quad -\Delta P_i = \frac{(B)}{(A)} \times 3.30 \times \frac{1.00 + 0.40}{0.60} \text{ m}\mu\text{moles}$$

The factor 3.30 is the total volume of the aqueous phase ($0.60 + 1.90 + 0.80 = 3.30$) in the extraction; the acetone added to the aqueous phase

in the first step is quantitatively extracted by the isobutanol-benzene phase.

The P:O ratio is then given by

$$\text{P:O} = \frac{-\Delta \text{ orthophosphate}}{-\frac{1}{2}\Delta \text{Fe}^{II} \text{ cytochrome } c} \tag{4}$$

Before the method of estimation of phosphate uptake described could be considered acceptable, it was subjected to critical tests, since it was necessary to be able to estimate precisely and accurately the uptake of quantities of only about 0.1 per cent of the inorganic phosphate pool present in the reaction medium. In the first place, completeness of extraction of labeled orthophosphate as phosphomolybdic acid into the isobutanol-benzene layer was essential. With the addition of carrier KH_2PO_4, which preceded each of the two extractions, the removal of orthophosphate from TCA-fixed enzyme reaction media of the type described was experimentally established to be complete to the approximate limit of the known radiochemical purity of the P^{32} supplied. The addition of acetone in the first step is essential for complete extraction of inorganic phosphate in the presence of relatively high concentrations of impure yeast hexokinase (and possibly other proteins added in excess). Although the acetone addition was found to be unnecessary for complete extraction from systems not containing hexokinase, it was employed as a routine in all determinations.

To ascertain whether organic phosphate is lost from the aqueous phase by extraction with isobutanol-benzene, samples of adenosine triphosphate (ATP) and glucose-6-phosphate labeled with P^{32} were tested; no significant loss was observed. The hydrolysis of ATP in all the media involved was less than 1 per cent in 10 minutes. It was also essential to establish that the volume of the aqueous phase did not change during extractions. Copper sulfate, which is insoluble in isobutanol-benzene-acetone mixtures, was added to the aqueous phase and its concentration determined spectrophotometrically as $Cu(NH_3)_4^{++}$ before and after the extraction. The concentration change was found to be less than 2 per cent, establishing that no significant change in the volume of the aqueous phase had occurred.

For each set of experiments a zero time determination of esterified P was carried out exactly as in the case of the incubated media to determine the unextractable base-line radioactivity corresponding approximately to the radiochemical impurities in the P^{32}. Such "base-line" radioactivity was subtracted from final counts in calculation of the P:O ratios. In most cases this blank was only about 10 per cent of the total measured radioactivity.

The separation procedure is sufficiently accurate to permit measurement of the uptake of as little as 0.25 mμmole of orthophosphate per ml. with a

precision of 10 per cent from a pool of 850 mμmoles per ml. The uptakes generally measured were considerably larger, and replicate analyses were within 5 per cent. With increasing orthophosphate concentration, however, the sensitivity becomes less. At 0.035 M phosphate, the uptake of 10 mμmoles of P represents the lower limit of sensitivity (10 per cent error). The method is thus considerably more sensitive than that described by Slater (11), particularly at the phosphate levels employed in this study. The probable error of the P:O ratios recorded is considered to be 10 per cent.

RESULTS AND DISCUSSION

The experimental methods described were used to measure directly the phosphorylation accompanying the oxidation of ferrocytochrome *c* to ferricytochrome *c* by rat liver mitochondria. In Table I are shown the experimental details and data from five such experiments. It is seen that the oxidation of ferrocytochrome *c* by mitochondria in the presence of molecular oxygen caused the uptake of labeled inorganic phosphate with observed P:O ratios as high as 0.86. The data of control experiments also recorded show that incubation of ferricytochrome *c* under the same conditions leads to relatively insignificant phosphate uptake. Similarly the complete omission of cytochrome *c* leads to insignificant phosphate uptake. In the absence of mitochondria the rate of autoxidation of ferrocytochrome *c* was negligible; no non-enzymatic phosphate uptake occurred (Experiment 1, Table I). The oxidation of ferrocytochrome *c* in these experiments occurs via cytochrome oxidase, since it was completely inhibited by low concentrations of cyanide; no phosphorylation was then observed (Experiment 1, Table I). The rate of oxidation declined with falling ferrocytochrome *c* concentration (Fig. 1). However, the P:O ratio was relatively constant with time, as is shown by the reaction curves in Fig. 1. At the end of this particular experiment about 60 per cent of the added ferrocytochrome *c* had been oxidized. From the data summarized in Table I and Fig. 1, it may be concluded that the phosphorylation is causally related to the enzymatic oxidation of ferrocytochrome *c*.

In these experiments ADP was added as the phosphate acceptor; ATP was therefore expected to be the isotopically labeled product of phosphorylation. This was established by paper chromatography of the deproteinized reaction medium from a phosphorylation experiment with no added Mg^{++} (see Table II) by the general method of Eggleston and Hems (12). The spot corresponding to ATP in the chromatogram contained approximately the expected amount of radioactivity; no radioactive spots corresponding to organic phosphate compounds other than adenosine polyphosphates were found on thorough examination of the paper.

For accurate measurement of the phosphorylation coupled to the oxidation of ferrocytochrome *c*, the presence of antimycin A in the reaction medium was essential. This antibiotic inhibited almost completely the reduction of ferricytochrome *c* by endogenous substrates of the mitochondria and also the phosphorylations coupled to such endogenous reduction

TABLE I

Phosphorylation Coupled to Oxidation of Ferrocytochrome c

Final concentrations in 1.0 ml. of reaction medium: 8.5×10^{-4} M orthophosphate labeled with P^{32} ($1.0 - 1.5 \times 10^3$ c.p.m. per mμmole P), 0.0015 M ADP, 0.020 M Tris buffer, pH 7.4, 0.001 M Versene, 0.005 M $MgCl_2$, and 1.65×10^{-5} M ferro- or ferricytochrome *c* as indicated below. The reaction was started by adding the mitochondria derived from 0.5 mg. wet weight of rat liver suspended in 0.20 ml. of 0.075 M sucrose + 0.001 M Versene containing 0.02 γ of antimycin A. Other details are given in the text. Fe^{II} = ferrocytochrome *c*, Fe^{III} = ferricytochrome *c*.

Experiment No.	Addition	Time	$-\frac{1}{4}\Delta Fe^{II}$	$-\Delta P_i$	P:O
		min.	*mμmoles*	*mμmoles*	
1	Fe^{II}	13.0	3.1	1.9	0.61
	" (no mitochondria)	13.0	0.0	0.0	
	" + 5×10^{-4} M KCN	13.0	−0.1	0.13	
	Fe^{III}	13.0		0.18	
	No cytochrome *c*	13.0		0.10	
2	Fe^{II}	14.5	2.9	2.5	0.86
	Fe^{III}	14.5		0.48	
3	Fe^{II}	2.2	1.0	0.68	0.68
	Fe^{III}	2.2		0.05	
4	Fe^{II}	11.7	4.3	2.0	0.47
	Fe^{III}	11.7		0.18	
5	Fe^{II}	2.3	1.0	0.53	0.53
	Fe^{III}	2.3		0.07	

of ferricytochrome *c* (see the following paper (13)). Antimycin A (0.02 γ per ml. in the standard test system) therefore permitted clear-cut experimental isolation of the respiratory chain between ferrocytochrome *c* and oxygen with little interference by electron flux from the preceding carriers. It should be pointed out, however, that in much higher concentrations (0.1 γ per ml.) antimycin A has the additional effect of inhibiting the phosphorylation coupled to the oxidation of ferrocytochrome *c* (Table II, Experiment 1).

The very dilute suspensions of mitochondria used are extraordinarily sensitive to traces of certain metal ions which uncouple phosphorylation. It was therefore necessary to use carefully cleaned glassware and frequently

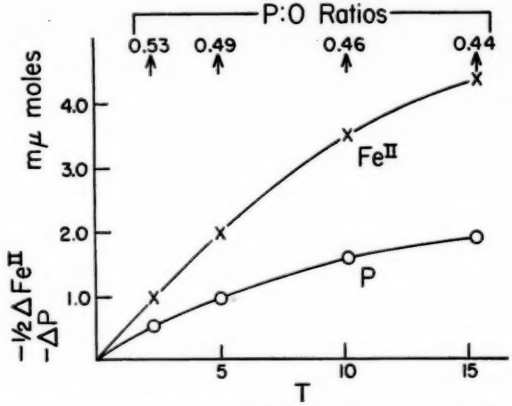


FIG. 1. Rates of phosphate uptake and oxidation of ferrocytochrome *c*. Final concentrations in reaction medium (total volume, 2.5 ml.) as in Table I. The reaction was started by addition of mitochondria at time zero, and 0.5 ml. aliquots were removed and fixed with 0.20 ml. of 25 per cent TCA + 0.05 M KH_2PO_4 at the times indicated and analyzed for phosphate uptake. The amounts of ferrocytochrome *c* (Fe^{II}) oxidized and orthophosphate taken up are given for a 1.0 ml. system.

TABLE II

Effect of Reaction Components on Coupled Phosphorylation

Components of complete system: 8.5×10^{-4} M orthophosphate labeled with P^{32} ($1.0 - 1.5 \times 10^3$ c.p.m. per mμmole of P), 0.0015 M ADP, 0.020 M Tris buffer, pH 7.4, 0.001 M Versene, 0.005 M $MgCl_2$, and 1.65×10^{-5} M ferrocytochrome *c*. The reaction was started in Experiments 2 and 3 by addition of mitochondria derived from 0.5 mg. wet weight of rat liver suspended in 0.20 ml. of 0.075 M sucrose + 0.001 M Versene containing 0.02 γ of antimycin A. In Experiment 1 the same amount of mitochondria suspension was added, but the concentration of antimycin A was varied as shown below.

Experiment No.	Reaction system	Time	$-\frac{1}{2}\Delta Fe^{II}$	$-\Delta P_i$	P:O
		min.	*mμmoles*	*mμmoles*	
1	Complete system + 0.006 γ antimycin A	14	4.2	2.3	0.55
	" " + 0.020 " " "		4.2	2.0	0.48
	" " + 0.10 γ antimycin A		4.8	0.91	0.19
2	" "	12	4.3	2.0	0.47
	" " (Versene omitted)		4.5	0.3	0.07
	" " (0.01 M NaF added)		3.9	1.9	0.49
	" " ($MgCl_2$ omitted)		3.7	1.3	0.35
3	" "	15	4.3	1.9	0.44
	" " (+ 2 mg. hexokinase and 20 μmoles glucose)		4.4	2.1	0.48

prepared reagent solutions throughout this study. Silica or Corex cuvettes with smooth internal surfaces were used; silica cuvettes with rough internal side walls which had been exposed to chromic acid were found useless, despite thorough washing (*cf.* Butler and Johnston (14)). Versene was found to be essential in the test medium for maximal P:O ratios (Table II); very little phosphorylation occurred in its absence. Neither *N*-hydroxyethylethylenediaminetriacetate ("Versenol," a chelating agent for trivalent metals) nor histidine could replace Versene.

Although considerable phosphorylation occurred in the absence of added Mg^{++}, demonstration of maximal P:O ratios required the presence of Mg^{++}

TABLE III

Uncoupling of Phosphorylation

Test conditions exactly as in Table I. Fe^{II} cytochrome *c* used as substrate throughout. Incubation, 13 to 16 minutes.

Experiment No.	Uncoupling agent	Concentration	$-\frac{1}{4}\Delta Fe^{II}$	$-\Delta P_i$	P:O
		M	*mμmoles*	*mμmoles*	
1	Control		3.1	1.9	0.61
	2,4-Dinitrophenol	1×10^{-4}	5.1	0.05	0.01
2	Control		4.5	2.3	0.51
	"Dicumarol"	1×10^{-5}	5.7	0.06	0.01
	Arsenate	2×10^{-3}	5.0	0.9	0.18
	Methylene blue	1×10^{-5}	4.2	0.9	0.21
	$CaCl_2$	2×10^{-3}	6.0	0.06	0.01
	Gramicidin	3×10^{-6}*	6.0	0.06	0.01

* Assuming molecular weight = 2800.

(Table II). Mn^{++} could not replace Mg^{++}, nor did Mn^{++} enhance the P:O ratios (*cf.* Lindberg and Ernster (15)).

The phosphorylation was found to be uncoupled by 2,4-dinitrophenol, gramicidin, arsenate, methylene blue, Ca^{++}, and dicumarol (Table III), agents which are already known to uncouple oxidative phosphorylations observed during oxidation of substrates such as glutamate, succinate, etc. It is significant that the rate of oxidation of ferrocytochrome *c* was increased in the presence of some of the uncoupling agents, indicating that the rate of oxidation of ferrocytochrome *c* in mitochondria is dependent on the availability of phosphate and phosphate acceptors (*cf.* Lardy and Wellman (16)). The uncoupling of phosphorylation by excess Ca^{++} and the protective action of Versene already noted in Table II are in agreement with the findings of Slater and Cleland (17) on heart sarcosomes. However, it cannot necessarily be concluded that the presence of Ca^{++} specifically is responsible for the Versene requirement.

An extended series of measurements of the P:O ratios made under the conditions described indicates values for the cytochrome-linked phosphorylation which are somewhat below those earlier observed when ascorbate was employed as the non-enzymatic reductant of ferricytochrome *c* in relatively large scale experiments (2, 3). Although some values approaching the expected limiting value of 1.0 were obtained, such as the value 0.86 shown in Table I, by and large most measurements fell in the range 0.4 to 0.6. The lower values could be the result of either dephosphorylation losses or inefficiency in the primary coupling mechanism, or a combination of these factors. From the following considerations, it appears unlikely that the lower P:O ratios are the result of dephosphorylation losses. Suitable control measurements of the rate of dephosphorylation of ATP by the very dilute mitochondrial suspensions used were carried out, and revealed

TABLE IV

Effect of Orthophosphate Concentrations

Experimental details as in Table I. Ferrocytochrome *c* used throughout as the substrate. Incubation, 13 minutes.

Phosphate concentration	$-\frac{1}{2}\Delta Fe^{II}$	$-\Delta P_i$	P:O
M	*mμmoles*	*mμmoles*	
6.2×10^{-5}	2.1	0.82	0.39
1.3×10^{-4}	2.4	1.02	0.43
3.4×10^{-4}	2.6	1.16	0.45
9.0×10^{-4}	2.9	1.5	0.52
5.3×10^{-3}	3.9	2.3	0.59

that at the extremely low concentrations of ATP generated in these phosphorylation tests the relative dephosphorylation loss is insignificant and unable to account for the deficit in the P:O ratios. This conclusion is supported by the finding that fluoride does not increase the P:O ratios, nor does the use of the hexokinase-glucose "trap" (Table II).

The relatively lower P:O ratios observed appear rather to be associated with suboptimal conditions for the primary coupling process. An important factor concerned appears to be the concentration of orthophosphate in the test medium. Although phosphorylation occurred at the lowest orthophosphate concentration tested (6.2×10^{-5} M), the P:O ratio gradually increases with orthophosphate concentration (Table IV). In addition, the rate of oxidation of ferrocytochrome *c* increased with increasing orthophosphate concentration. Unfortunately it was not possible to obtain accurate measurements of the P:O ratios at phosphate concentrations substantially above 0.0053 M orthophosphate because the radiochemical impurities in the P^{32} imposed an upper limit. However, by extrapolation

of the data in Table III, the P:O ratio could be expected to be approximately 1.0 at orthophosphate concentrations of about 0.03 to 0.04 M. Slater and Cleland have already demonstrated that maximal P:O ratios occurred only at very high orthophosphate concentrations (0.03 to 0.04 M) in the case of the oxidation of α-ketoglutarate (18); the findings presented here indicate that the cytochrome-linked phosphorylation may have the same property. Most of the P:O ratios recorded in this study have therefore been measured under suboptimal phosphate concentrations.

It appears likely that the second major factor involved in the lower P:O ratios observed with the direct method is the uncoupling effect of what appear to be trace metal contaminants. Although the presence of Versene appears to abolish most of this effect, it is uncertain whether it affords complete protection.

These factors (*i.e.*, suboptimal orthophosphate concentration and the possible presence of uncoupling metal ions) appear to rule against observation of maximal P:O ratios under the conditions used. The hypotonic pretreatment of the mitochondria, which is necessary for external ferrocytochrome *c* to react with the oxidizing sites in the mitochondria (2, 6), is also a factor ruling against the demonstration of true maximal P:O ratios. The tonicity used (0.075 M sucrose) was found to be optimal but probably results in some damage to the phosphorylating enzyme system. It is therefore not unreasonable to conclude that the data and considerations presented are consistent with the P:O ratios obtained earlier with ascorbate as reductant (2, 3), which approached the value 1.0.

The rate of oxidation of ferrocytochrome *c* by the mitochondria was obviously limited by factors of permeability or "accessibility" previously discussed (2, 6). A first order dependence of rate on concentration of ferrocytochrome *c* was observed, and at the low concentrations employed the oxidase was far from "saturated." The rate of oxidation (Q_{O_2} = microliters of O_2 uptake per mg. dry weight of mitochondria per hour at 37°) under these conditions was found to be about 40 to 50.

Mitochondria isolated from rat kidney, heart, and brain also showed the existence of the phosphorylation coupled to oxidation of ferrocytochrome *c* (Table V). In these tests, no attempt was made to establish optimal experimental conditions for each type of mitochondria; it appears certain that these conditions might be somewhat different for each type. The P:O ratios observed were low but give unmistakable qualitative evidence that this phosphorylation occurs in the preparations tested.

An exchange reaction between labeled orthophosphate and ATP not dependent on electron flux has been observed in suspensions of mitochondria by Boyer *et al.* (19). It is appropriate to consider the possibility that the method of measuring phosphate uptake used in this study is subject to

significant interference from this reaction. However, it is already quite clear from the data in Table I that the phosphate uptake occurs only during oxidation of ferrocytochrome *c*. In the absence of added cytochrome *c*, only negligible "base-line" phosphorylation occurred. The exchange reaction described by Boyer *et al.* (19) can, however, be observed in very dilute suspensions of mitochondria, but only if the ratio of ATP to ADP in the test medium is quite high.[1] At the low ATP to ADP ratios prevailing in the P:O tests described, the rate of the exchange reaction is insignificantly low. Further evidence of the validity of the method used is provided by a comparison of the P^{32} method with simple colorimetric estimation of the net phosphate uptake in a macro experiment with ascorbic acid

TABLE V

Cytochrome-Linked Phosphorylation in Rat Tissues

The mitochondria were isolated by the general method used for liver and suspended in 0.075 M sucrose-0.001 M Versene in such a concentration to yield appropriate rates of oxidation of ferrocytochrome *c* in the test. The test conditions were otherwise identical to those shown in Table I.

Source of mitochondria	Substrate	$-\frac{1}{4}\Delta Fe^{II}$	$-\Delta P_i$	P:O
		mμmoles	*mμmoles*	
Kidney	Fe^{II}	3.8	0.69	0.18
	Fe^{III}		0.23	
Heart	Fe^{II}	3.0	0.71	0.24
	Fe^{III}		0.20	
Brain	Fe^{II}	3.4	1.20	0.35
	Fe^{III}		0.08	

as non-enzymatic reductant of cytochrome *c* (Table VI). In the presence of the hexokinase-glucose "trap," which insures a low ATP:ADP ratio, the isotopic method gives the same value for phosphate uptake as the measurement of net uptake. In the absence of the hexokinase-glucose trap, however, the P^{32} method overestimated phosphate uptake by some 17 per cent. However, in the latter case the ratio of ATP to ADP was very much higher than in the system containing hexokinase, and the exchange reaction therefore became a significant interfering factor in the use of the P^{32} method. Although the P^{32} method of estimating phosphate uptake appeared to be the only one sensitive enough to measure the very minute changes involved in this study, it is obvious that it must be used with some appreciation of the possible complications.

The existence of one phosphorylation coupled to the oxidation of 2 moles of ferrocytochrome *c*, indicated by the direct measurements recorded here

[1] Unpublished observations.

and by the earlier indirect measurements (2, 3), is completely compatible with existing data on phosphorylating sites and with known thermodynamic relationships in the respiratory chain. The over-all equation for the oxidation of ferrocytochrome *c* in phosphorylating mitochondria can thus be written

$$2Fe^{II} \text{ cytochrome } c + P + ADP + 2H^+ + \tfrac{1}{2}O_2 \rightarrow$$

$$2Fe^{III} \text{ cytochrome } c + ATP + H_2O$$

Under standard conditions the oxidation of 2 moles of ferrocytochrome *c*

TABLE VI

Comparison of Isotopic and Colorimetric Methods

Final concentrations in medium (total volume, 2.0 ml.): 0.010 M orthophosphate, labeled with P^{32} (1.39×10^4 c.p.m. per μmole P), 0.0025 M ADP, 0.01 M Tris buffer, pH 7.4, 0.001 M Versene, 0.005 M $MgCl_2$, 0.01 M KF, 1×10^{-5} M cytochrome *c*, 0.05 M ascorbate, and, when present, 3.5 mg. of hexokinase per ml. and 0.03 M glucose. In the tests carried out in the absence of hexokinase and glucose, the ADP concentration was increased to 0.0050 M. The reaction was started by adding the mitochondria derived from 0.5 gm. wet weight of rat liver suspended in 0.5 ml. of 0.075 M sucrose + 0.001 M Versene. Oxygen uptake measured manometrically at 15°. Reaction time, 20 minutes.

System	Oxygen uptake	P uptake		P:O
		Colorimetric	Isotopic	
	μatoms	*μmoles*	*μmoles*	
Ascorbate + hexokinase-glucose trap	8.58	8.21		0.96
			7.99	0.93
Ascorbate; ADP as acceptor; no trap	7.57	3.27		0.43
			3.79	0.50

($E'_0 = +0.26$ at pH 7.4) by 0.5 mole of oxygen would result in a free energy change of −26,200 calories. If the free energy required for synthesis of 1 mole of ATP from orthophosphate and ADP is assumed to be 12,000 calories (*cf.* Burton and Krebs (20)), then it is seen that the coupled phosphorylation recovers approximately 46 per cent of the free energy loss in the oxidative reaction. Earlier work has revealed that probably three phosphorylations are coupled to the passage of a pair of electrons from reduced diphosphopyridine nucleotide to oxygen (6); the results reported here provide direct evidence that one of these phosphorylations occurs in that portion of the respiratory chain between cytochrome *c* and oxygen.

The data obtained in this study do not permit any conclusions regarding the mechanism of the phosphorylation coupled to the transport of electrons from cytochrome *c* to oxygen. However, it appears unlikely that the phos-

phorylation is coupled to the reduction of ferricytochrome *a* by ferrocytochrome *c*, since the standard oxidation-reduction potential of cytochrome *c* is about +0.26 volt and that of cytochrome *a* about +0.29 volt (21). It is therefore more likely that it is coupled to the span between ferrocytochrome *a* and oxygen.

SUMMARY

The oxidation of small amounts of ferrocytochrome *c*, added as substrate to very dilute suspensions of rat liver mitochondria supplemented with orthophosphate, ADP, and dissolved oxygen, caused a coupled phosphorylation of the ADP to ATP. The rate of oxidation was followed spectrophotometrically, and the phosphorylation by a sensitive method with P^{32} as tracer. The P:O ratios observed (highest value, 0.86) are consistent with earlier measurements with ascorbic acid as non-enzymatic reductant of cytochrome *c* and indicate that 1 mole of ATP is formed from ADP and orthophosphate per 2 moles of ferrocytochrome *c* oxidized. Over the reaction times tested the P:O ratio was relatively constant with time. The P:O ratio increased with orthophosphate concentration. The phosphorylation was found to be uncoupled by 2,4-dinitrophenol, Ca^{++}, arsenate, dicumarol, and methylene blue. Both oxidation and phosphorylation were abolished in the presence of cyanide. Exchange reactions between orthophosphate and ATP independent of electron flux were shown not to interfere in the phosphorylation measurements with P^{32}. It is concluded that one of the three phosphorylations coupled to transport of a pair of electrons from diphosphopyridine nucleotide to oxygen occurs in the span between cytochrome *c* and oxygen.

The patient and skilful technical assistance of Dorothy Jacobs is gratefully acknowledged.

BIBLIOGRAPHY

1. Nielsen, S. O., and Lehninger, A. L., *J. Am. Chem. Soc.*, **76,** 3860 (1954).
2. Lehninger, A. L., ul Hassan, M., and Sudduth, H. C., *J. Biol. Chem.*, **210,** 911 (1954).
3. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **210,** 903 (1954).
4. Nason, A., Wosilait, W. D., and Terrell, A. J., *Arch. Biochem. and Biophys.*, **48,** 233 (1954).
5. Kern, M., and Racker, E., *Arch. Biochem. and Biophys.*, **48,** 235 (1954).
6. Lehninger, A. L., *Harvey Lectures*, **49,** 176 (1953–54).
7. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39,** 289 (1945).
8. Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21,** 965 (1949).
9. Theorell, H., and Åkesson, Å., *J. Am. Chem. Soc.*, **63,** 1804, 1812 (1941).
10. Ernster, L., Zetterström, R., and Lindberg, O., *Acta chem. Scand.*, **4,** 942 (1950).
11. Slater, E. C., *Biochem. J.*, **53,** 521 (1953).

12. Eggleston, L. V., and Hems, R., *Biochem. J.*, **52,** 156 (1952).
13. Borgström, B., Sudduth, H. C., and Lehninger, A. L., *J. Biol. Chem.*, **215,** 571 (1955).
14. Butler, E. B., and Johnston, W. H., *Science*, **120,** 543 (1954).
15. Lindberg, O., and Ernster, L., *Nature*, **173,** 1038 (1954).
16. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195,** 215 (1952).
17. Slater, E. C., and Cleland, K. W., *Biochem. J.*, **55,** 566 (1953).
18. Slater, E. C., and Cleland, K. W., *Biochem. J.*, **53,** 557 (1953).
19. Boyer, P. D., Falcone, A. B., and Harrison, W. H., *Nature*, **174,** 401 (1954).
20. Burton, K., and Krebs, H. A., *Biochem. J.*, **54,** 94 (1953).
21. Ball, E. G., *Biochem. Z.*, **295,** 262 (1938).

PHOSPHORYLATION COUPLED TO REDUCTION OF CYTOCHROME *c* BY β-HYDROXYBUTYRATE*

By BENGT BORGSTRÖM,† HERSCHEL C. SUDDUTH,‡ AND ALBERT L. LEHNINGER

(From the Department of Physiological Chemistry, School of Medicine, The Johns Hopkins University, Baltimore, Maryland)

(Received for publication, November 12, 1954)

Of the three phosphorylations coupled to the passage of a pair of electrons from DPNH[1] to oxygen in the respiratory chain of mitochondria (1–3), one has been definitely established to be coupled to the oxidation of ferrocytochrome *c* by molecular oxygen (4–6). Although thermodynamic considerations indicate that the other two phosphorylations occur between DPNH and cytochrome *c* (3), little direct experimental evidence is available. It has been demonstrated that phosphorylation occurs during the oxidation of various substrates by mitochondria when ferricyanide is used as an electron acceptor (2, 7, 8). Since the identity of the respiratory carriers reacting with the ferricyanide in such experiments is not known, such data do not establish the phosphorylation sites in the respiratory chain with certainty. However, with this method Copenhaver and Lardy (2) have found that one phosphorylation probably occurs between β-hydroxybutyrate and the antimycin A-sensitive point in the respiratory chain (presumably the step immediately involved in reduction of ferricytochrome *c*).

A more direct approach, namely the use of added ferricytochrome *c* as electron acceptor, has been briefly reported by Slater (9), who measured the phosphorylation coupled to the oxidation of α-ketoglutarate in heart sarcosome suspensions. The P:O ratios observed for the α-ketoglutarate-ferricytochrome *c* reaction were between 1.29 and 2.20. However, the meaning of these figures is not entirely clear because of the existence of a substrate-linked phosphorylation coupled to α-ketoglutarate oxidation

* Supported by grants from the National Institutes of Health and the Nutrition Foundation, Inc.

† Fellow of the Rockefeller Foundation. Present address, University of Lund, Lund, Sweden.

‡ Commander, Medical Corps, United States Navy. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

[1] The following abbreviations are used in this paper: DPNH, reduced diphosphopyridine nucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Versene, ethylenediaminetetraacetate; CoA, coenzyme A.

(*cf.* Kaufman *et al.* (10)) and because heart sarcosomes, as studied in Slater's laboratory, have not yielded as high P:O ratios for the α-ketoglutarate-oxygen reaction (11) as have been reported by others (12, 13).

This report describes direct measurements of the phosphorylation coupled to electron transport between β-hydroxybutyrate and added ferricytochrome *c* in suspensions of rat liver mitochondria. The methods outlined in the preceding paper were used (4). Cytochrome oxidase was blocked with cyanide to prevent reoxidation of the ferrocytochrome *c* formed. The P:O ratios observed are consistent with the existence of two phosphorylations coupled to the passage of a pair of electrons from β-hydroxybutyrate to ferricytochrome *c*.

Methods and Materials

The spectrophotometric measurement of reduction of ferricytochrome *c*, the isotopic analysis of phosphorylation, and the preparation of rat liver mitochondria were carried out by the methods described in detail in the preceding paper (4).

RESULTS AND DISCUSSION

For the phosphorylation tests, suspensions of mitochondria were incubated in cuvettes containing β-hydroxybutyrate, ferricytochrome *c*, orthophosphate (P_i) labeled with P^{32}, ADP, $MgCl_2$, Versene, fluoride, KCl, and cyanide. The reduction of ferricytochrome *c* was followed spectrophotometrically at 550 mμ, and the phosphate uptake measured by the isotopic method. Enzymatic reoxidation of the ferrocytochrome *c* produced was prevented by the presence of cyanide in the medium to inhibit cytochrome oxidase. The P:O ratio was calculated from the expression

$$\text{(1)} \qquad \text{P:O} = \frac{-\Delta \text{ inorganic P (m}\mu\text{moles)}}{-\frac{1}{2}\Delta \text{Fe}^{\text{III}} \text{ cytochrome } c \text{ (m}\mu\text{moles)}}$$

The observed P:O ratios have a probable error no larger than 10 per cent (4). The rate of reduction of ferricytochrome *c* by β-hydroxybutyrate in such systems was found to be nearly linear over a period of 20 minutes (Fig. 1).

The results of a series of tests on rat liver mitochondria are shown in Table I. It is seen that the P:O ratios observed fell in the range, 1.10 to 1.93, with the average of 1.51. Although low values (<1.0) were observed in the earlier phases of the study, after appropriate reaction conditions were established, the P:O ratios were consistently and significantly above 1.0. No values significantly above 2.0 were observed. It has already been shown that the concentration of orthophosphate in the medium is a very important factor in observing maximal P:O ratios in this type of experiment (4, 14); in this study considerably less than optimal concentrations

of phosphate was used, a fact which probably accounts for at least some of the deficit between the observed average of 1.51 and the theoretically expected value 2.0. In any case, a sufficient number of measurements have been made as described to warrant the conclusion that two phosphorylations are coupled to the passage of electrons along the respiratory chain from β-hydroxybutyrate to ferricytochrome *c* in the type of preparations tested.

A complicating factor in these measurements is the fact that considerable endogenous reduction of ferricytochrome *c* takes place in the absence of added substrates (Table I and Fig. 1). The electron donors are not known, but are presumably tricarboxylic acid cycle intermediates, etc.

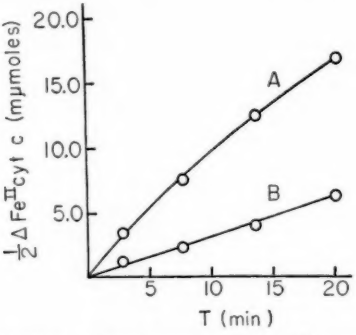


FIG. 1. Rate of reduction of ferricytochrome *c*. The experimental conditions were typical of those employed in the experiments described in Table I. Curve A represents the rate of reduction when β-hydroxybutyrate was the substrate; Curve B represents the rate of reduction in the absence of added substrate.

Accompanying this endogenous reduction of ferricytochrome *c* is some coupled phosphorylation. This endogenous reduction and phosphorylation made up from 8 to 47 per cent of the total reduction observed in the presence of substrate in the various experiments shown in Table I. The average endogenous fraction was 30 per cent. The P:O of this endogenous reduction was found to be about the same as that observed for the complete system in most cases, as is seen in Table I, and it is therefore felt that no serious error is caused by the endogenous component. In an effort to find appropriate experimental material with negligible endogenous activity, mitochondria isolated from rat kidney and brain and various mouse tissues were examined. All showed coupled phosphorylation, but in each case the endogenous component was as high as in the liver mitochondria. Fasting of the animals did not always cause significant decreases in this endogenous reduction.

A 15 minute preincubation of rat liver mitochondria at 0° in 0.18 M su-

crose was found optimal for observation of the coupled phosphorylation. Preincubation at lower sucrose concentrations caused preparations to show

TABLE I

Phosphorylation Coupled to β-Hydroxybutyrate-Ferricytochrome c Reaction

Test cuvettes contained, in 1.0 ml. total volume, from 4 to 5 × 10^{-5} M ferricytochrome *c*, orthophosphate labeled with P^{32} in concentrations indicated, 0.002 M Versene, 0.0025 M ADP, 0.145 M KCl, 0.01 M glycylglycine buffer, pH 7.4, 0.005 M $MgCl_2$, 0.01 M KF, 0.0005 M KCN, 0.01 to 0.03 M *dl*-β-hydroxybutyrate (BOH). Rat or mouse liver or kidney mitochondria were first preincubated in hypotonic sucrose solutions (0.075 to 0.18 M) for 5 to 15 minutes at 0°, so that 1.0 ml. contained the mitochondria derived from 10 to 30 mg. of whole tissue. Reaction started by adding the preincubated mitochondria to the test cuvette; the final concentrations of mitochondria were the equivalent of 2.5 to 7.5 mg. of fresh whole tissue per ml.; 23–26°; 14 to 25 minutes. Separate preparations of mitochondria used for each experiment. L., liver; K., kidney; R., rat; M., mouse. The P:O ratios for endogenous activity are given in parentheses.

Experiment No.		Substrate	Ortho-phosphate	$-\frac{1}{4}\Delta Fe^{III}$ cytochrome *c*	$-\Delta P_i$	P:O
			M	*mμmoles*	*mμmoles*	
1	R. L.	None	0.00174	5.55	8.14	(1.46)
		BOH	0.00174	13.2	21.2	1.61
		"	0.00174	17.7	22.0	1.24
2	R. L.	None	0.00174	3.1	5.6	(1.81)
		BOH	0.00174	11.7	14.6	1.25
3	R. L.	None	0.00174	2.7	1.3	(0.48)
		BOH	0.00174	13.9	15.3	1.10
4	R. L.	"	0.00105	7.1	11.0	1.55
5	R. K.	"	0.00130	15.5	26.7	1.72
		"	0.00130	15.8	29.3	1.85
6	R. K.	None	0.00130	4.5	8.2	(1.82)
		BOH	0.00130	17.9	21.5	1.20
7	R. K.	None	0.00130	0.84	1.5	(0.58)
		BOH	0.00130	16.0	18.6	1.16
8	M. L.	None	0.00105	2.3	3.5	(1.52)
		BOH	0.00105	11.9	15.4	1.29
9	R. L.	None	0.0054	3.7	7.2	(1.95)
		BOH	0.0054	10.5	20.2	1.92
10	R. L.	"	0.0054	9.0	17.4	1.93
		"	0.0054	9.4	17.7	1.88
		" (Fe^{III} cytochrome *c* omitted)	0.0054		2.0	
11	R. L.	None	0.00174	5.8	9.5	(1.64)
		BOH	0.00174	13.9	22.6	1.63
12	R. L.	None	0.00174	5.5	9.7	(1.76)
		BOH	0.00174	15.1	20.5	1.36

a greatly increased rate of reduction, but the P:O ratio declined. At higher sucrose concentrations the rate of reduction of cytochrome *c* was

greatly diminished and the endogenous component became higher. These findings probably are a reflection of the permeability of the mitochondrial membrane to external cytochrome *c* under conditions of varying tonicity (1, 3, 5). Complete omission of the ferricytochrome acceptor reduced phosphate uptake to the endogenous level or less (Table I, Experiment 10).

The phosphorylation associated with this portion of the respiratory chain was found to be uncoupled by 2,4-dinitrophenol, Ca^{++}, and gramicidin (Table II) without greatly affecting the rate of reduction of cytochrome *c*. These findings are thus in essential agreement with similar observations when oxygen was used as acceptor and with the cytochrome-linked phosphorylation already described (4).

TABLE II

Effect of Inhibitors

Test conditions as in Table I. The substrate was β-hydroxybutyrate in all cases. Mouse liver mitochondria in Experiment 1; rat liver in Experiment 2.

Experiment No.	Inhibitor	$-\frac{1}{2}\Delta Fe^{III}$ cytochrome *c*	$-\Delta P_i$	P:O
		mμmoles	*mμmoles*	
1	None	13.5	12.9	0.95
	Antimycin A, 0.02 γ per ml.	1.7	1.9	
	" " 0.20 " " "	1.5	1.6	
2	None	10.9	16.3	1.49
	2,4-Dinitrophenol, 1×10^{-4} M	11.4	0.92	0.08
	$CaCl_2$, 0.005 M	8.4	0.90	0.11
	Gramicidin, 20 γ per ml.	8.8	1.43	0.16

It has been demonstrated that antimycin A almost completely inhibits electron transport in intact phosphorylating mitochondria (2, 15), presumably at a point between DPN-cytochrome reductase and cytochrome *c*. In agreement with these findings, it was found that antimycin A[2] virtually completely inhibited the reduction of cytochrome *c* by β-hydroxybutyrate in the systems described, as well as the phosphorylation (Table II).

Although β-hydroxybutyrate was used as the substrate in most of the tests carried out, phosphorylation was also observed with glutamate, malate, lactate, succinate, and α-ketoglutarate as substrates. Some of these data are shown in Table III. The results are in general agreement with predictions: glutamate yielded P:O ratios equivalent to those obtained with β-hydroxybutyrate, as would be expected, since both substrates are DPN-linked. In the case of succinate, rather low P:O ratios (0.37) were observed; actually, a P:O ratio of 1.0 is the expected maximum if one of the two phosphorylations between succinate and oxygen (16) is linked to

[2] The antimycin A was a generous gift of Professor Frank M. Strong, University of Wisconsin.

the step between cytochrome *c* and oxygen (4). However, no special effort was made to establish optimal conditions for each of these substrates. These experimental conditions were found to be unsuitable for accurate measurement of phosphorylation coupled to the reduction of ferricytochrome *c* by α-ketoglutarate. This substrate forms a cyanohydrin, thus removing a large part of the HCN present in the medium. Cytochrome oxidase activity was therefore incompletely inhibited by low concentrations of cyanide in the presence of α-ketoglutarate. Although cyanide forms a complex with cytochrome *c* (17), the rate of this reaction under the conditions described was relatively very low and did not limit the rate of reduction of cytochrome *c* in the short time intervals studied.

TABLE III

P:O Ratios with Various Substrates

Experimental details as in Table I. Concentration of Fe^{III} cytochrome *c* = 4.37 × 10^{-5} M, of orthophosphate 1.05 × 10^{-3} M. Time, 20 minutes. Mitochondria pretreated 5 minutes at 0° in 0.15 M sucrose. Cuvettes with β-hydroxybutyrate, glutamate, and succinate contained the mitochondria derived from 2.5 mg. of whole mouse liver per ml.; those with succinate, mitochondria from 0.75 mg. of whole liver per ml.

Substrate	$-\frac{1}{2}\Delta Fe^{III}$ cytochrome *c*	$-\Delta P_i$	P:O
	mμmoles	*mμmoles*	
β-Hydroxybutyrate, 0.025 M	11.9	15.4	1.29
Glutamate, 0.01 M	13.7	16.8	1.23
" 0.01 "	13.7	9.3	1.38
Succinate, 0.01 M	11.3	4.1	0.36
" 0.01 "	11.3	4.2	0.37

The observed P:O ratios permit the conclusion that two phosphorylations occur on passage of a pair of electrons from DPNH to ferricytochrome *c* (3). From the standard potentials of the two systems, $E'_0 = -0.32$ volt and $E'_0 = +0.26$ volt (pH 7.0) respectively, the standard free energy change in the over-all reaction is approximately −26,800 calories per mole. Assuming a value of 12,000 calories per mole for the increase in free energy accompanying formation of 2 moles of ATP from ADP and phosphate, the phosphorylation mechanisms are approximately 89 per cent efficient in energy recovery in the complete process. The over-all reaction may thus be written

(2) $DPNH + H^+ + 2P + 2ADP + 2Fe^{III}$ cytochrome $c \rightarrow$

$DPN^+ + 2ATP + 2Fe^{II}$ cytochrome *c*

More precise localization of the two phosphorylations is not yet possible.

However, Copenhaver and Lardy have made the important finding that one of these probably occurs between DPNH and the antimycin-sensitive step (2). On the basis of this observation and on thermodynamic grounds, it appears likely that one is coupled to the interaction of DPNH and the flavoprotein cytochrome reductase and the other to the interaction of the latter with cytochrome *c* (3).

SUMMARY

Enzymatic reduction of ferricytochrome *c* by β-hydroxybutyrate in suspensions of mitochondria supplemented with labeled orthophosphate, ADP, Mg^{++}, and Versene was accompanied by coupled phosphorylation of the ADP. The reoxidation of ferrocytochrome *c* was prevented by inhibiting cytochrome oxidase with cyanide. The test system thus isolated that portion of the respiratory chain between the substrate and cytochrome *c*. From measurements of the amount of ferricytochrome reduced and the orthophosphate taken up, the observed P:O ratios in a series of experiments fell in the range from 1.10 to 1.93, with an average of 1.51. The data are consistent with the conclusion that two phosphorylations are coupled to the passage of a pair of electrons from DPNH to cytochrome *c*, in agreement with other experimental findings and with thermodynamic considerations. The phosphorylations were uncoupled by 2,4-dinitrophenol, gramicidin, and $CaCl_2$.

BIBLIOGRAPHY

1. Lehninger, A. L., *J. Biol. Chem.*, **190,** 345 (1951).
2. Copenhaver, J. H., Jr., and Lardy, H. A., *J. Biol. Chem.*, **195,** 225 (1952).
3. Lehninger, A. L., *Harvey Lectures*, **49,** 176 (1953–54).
4. Nielsen, S. O., and Lehninger, A. L., *J. Biol. Chem.*, **215,** 555 (1955).
5. Lehninger, A. L., ul Hassan, M., and Sudduth, H. C., *J. Biol. Chem.*, **210,** 911 (1954).
6. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **210,** 903 (1954).
7. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177,** 655 (1949).
8. Judah, J. D., *Biochem. J.*, **49,** 271 (1951).
9. Slater, E. C., *Nature*, **166,** 982 (1950).
10. Kaufman, S., Gilvarg, C., Cori, O., and Ochoa, S., *J. Biol. Chem.*, **203,** 869 (1953).
11. Slater, E. C., and Holton, F. A., *Biochem. J.*, **56,** 28 (1954).
12. Maley, G. F., and Plaut, G. W. E., *J. Biol. Chem.*, **205,** 297 (1953).
13. Lardy, H., and Copenhaver, J. H., Jr., *Nature*, **174,** 231 (1954).
14. Slater, E. C., and Cleland, K. W., *Biochem. J.*, **53,** 557 (1953).
15. Potter, V. R., and Reif, A. E., *J. Biol. Chem.*, **194,** 287 (1952).
16. Hunter, F. E., Jr., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 297 (1950).
17. Horecker, B. L., and Kornberg, A., *J., Biol. Chem.*, **165,** 11 (1946).

THE ACTION OF PANCREATIC DEOXYRIBONUCLEASE*

II. ISOMERIC DINUCLEOTIDES

By ROBERT L. SINSHEIMER

(From the Department of Physics, Iowa State College, Ames, Iowa)

(Received for publication, November 15, 1954)

In Paper I (1) methods were described for the isolation of dideoxyribonucleotides from digests of deoxyribonucleic acid (DNA) by pancreatic deoxyribonuclease (DNase). Dinucleotides composed of unlike mononucleotides were obtained as mixtures of the two possible sequential isomers. Methods have now been developed for the analysis of the proportions of the isomers in such mixtures and applied to the dinucleotide fractions obtained by the action of DNase on DNA from calf thymus.

Material and Methods

Dinucleotide fractions were obtained as described previously (1). Purified venom phosphodiesterase was prepared as before (2). Prostatic phosphomonoesterase was generously supplied by Dr. Gerhard Schmidt. All fractionations were carried out on Dowex 1-8X anion exchange resin.

Principles of Isomer Analysis—The method consists of the following steps: (*a*) removal of the terminal phosphate groups of the mixed isomeric dinucleotides with prostatic phosphomonoesterase; (*b*) isolation of the resultant mixed isomeric dinucleotide phosphates by ion exchange chromatography; (*c*) splitting of the mixed isomeric dinucleotide phosphates by purified venom phosphodiesterase; (*d*) recovery of the mononucleotide fractions from this latter digest by ion exchange chromatography. From the optical density of each nucleotide fraction and the extinction coefficients (1), the molar ratio ($X:Y$) of the two mononucleotides (X and Y) can be calculated. This molar ratio ($X:Y$) is then taken to be the molar ratio of the dinucleotide isomers containing X and Y as their initial nucleotides (X-Y/Y-X).

This conclusion is based upon the assumptions that the terminal phosphate of the dinucleotides is attached to a 5′ position and that the venom phosphodiesterase always splits the 5′-3′ phosphate diester link at the 3′ end. The validity of these assumptions is supported by the following observations.

The mononucleotides obtained by venom phosphodiesterase digestion of a DNase digest are completely dephosphorylated by 5-nucleotidase of

* The research described in this paper was supported by a grant from the United States Public Health Service.

XUM

snake venom ((3), and personal observation). Since this enzyme will not attack 3′-deoxyribonucleotides (4), the terminal phosphate groups of all the polynucleotide fractions must be attached at the 5′ position, and the phosphodiesterase must always attack at the 3′ end of the 5′-3′ phosphate diester.

TABLE I

Strength of Acetate Buffer (pH 4.3) Employed to Elute Dinucleotide Phosphates

Dinucleotides*	Molarity of buffer of pH 4.3
CT	0.04
CA	0.05
MG	0.16
CG	0.18
TA	0.20
TG	0.35
AG	0.35

* C = deoxycytidylic acid; T = thymidylic acid; A = deoxyadenylic acid; M = 5-methyldeoxycytidylic acid; G = deoxyguanylic acid.

TABLE II

Molar Ratios of Mononucleotides Obtained by Phosphodiesterase Degradation of Dinucleotide Phosphates

Dinucleotide	Molar ratio
CT	T:C = 3.0
CA	A:C = ∞
TA	A:T = 13.3
CG	C:G = 5.4
MG	G:M = ∞
TG	G:T = 15.7
AG	A:G = 4.9

Similarly, if the pooled dinucleotide fractions (D-1, D-2, and D-3; see (1)) from a DNase digest are treated with prostatic phosphomonoesterase to remove all terminal phosphate groups and then, after inactivation of the prostate enzyme,[1] are treated with rattlesnake venom, which contains phosphodiesterase and 5-nucleotidase (3, 6), the entire digest is converted to nucleosides. The phosphodiesterase must again attack only at the 3′

[1] The prostatic phosphomonoesterase may be inactivated by shaking the solution with ether for 30 minutes at room temperature (5) (this procedure leaves less than 5 per cent of the original activity) and, further, by adjustment of the pH to 9.2 for the snake venom action, which reduces the residual prostate enzyme activity below detectable levels (less than 0.01 per cent of the original activity).

end of the 5′-3′ phosphate diester link in the dinucleotides without terminal phosphate groups.

Technique of Isomer Analysis—125 to 250 γ of dinucleotide, in a volume of approximately 100 μl., are added to 1 ml. of 0.2 M acetate buffer, pH 5.5. 50 μl. of the prostatic phosphatase preparation are added, and the solution is incubated at 37° for 4 hours. The digest is then made alkaline by addition of 1 N NH_4OH and added to a column of resin, 10 cm. long × 3 mm. in diameter. An appropriate strength (Table I) of ammonium acetate buffer, pH 4.3, is then used to elute the desired dinucleotide phosphate from the column. The fractions containing the eluted substance, detected by its ultraviolet absorption, are pooled and lyophilized to remove water and salt.

TABLE III

Molar Proportions of Dinucleotides in Thymus DNA Digest

XY = X-p-Y-p.*

x \ y	M	C	T	A	G
M	0		0	0	0
C		1.11	0.78	0	0.75
T	0	2.34	1.38	0.10	0.16
A	0	3.22	1.36	0.46	0.97
G	1.03	0.12	2.61	0.20	0.82

* *Y* refers to the nucleoside carrying the phosphate monoester group in each case.

The dinucleotide phosphate is then taken up in 1 ml. of 0.2 M NH_4OH-NH_4 acetate buffer, pH 9.2. To this are added 0.2 ml. of 0.3 M magnesium acetate and 50 to 100 μl. of purified venom phosphodiesterase. The digest is incubated at 37° for 4 hours and then added to a column of resin, 10 cm. long × 3 mm. in diameter. The mononucleotides are analyzed as described previously (7, 8).

Results

The molar ratios of the nucleotides found in the phosphodiesterase digests of the dinucleotide phosphate fractions, obtained as above from a DNase digest of calf thymus DNA, are presented in Table II. These values are in each case the averages of two or more determinations made upon dinucleotide fractions prepared at different times from different DNase digests. The variations in isomer ratios of fractions from different digests have in all instances been less than ±3 per cent.

From these ratios, knowing the molar fractions corresponding to each of the mixed isomers in the DNase digest (Table III in Sinsheimer (1)),

we have calculated the molar fraction of each dinucleotide isomer in the digest (Table III).[2]

DISCUSSION

The only previous report of the analysis of a dideoxyribonucleotide relative to its isomeric composition has been the finding of Smith and Markham (9) that the dinucleotide fraction containing deoxyadenylic and deoxycytidylic acids in a DNase digest of calf thymus DNA was entirely of the sequence adenylic-cytidylic. Our results confirm this report.

The most striking result of the complete isomer analysis is to be found among the dinucleotides composed of one purine and one pyrimidine nucleotide. Here, with one exception, the dinucleotides with the sequence purine nucleotide-pyrimidine nucleotide are in great excess over those with the sequence pyrimidine nucleotide-purine nucleotide. In fact, two of the latter type isomers (cytidylic-adenylic and 5-methylcytidylic-guanylic) could not be detected. The single exception is the dinucleotide with cytidylic and guanylic acids, in which the sequence cytidylic-guanylic is over 5 times as abundant as its isomer.

That the dinucleotide of 5-methylcytidylic and guanylic acids should be all of the sequence purine nucleotide-pyrimidine nucleotide, while that of cytidylic and guanylic acids should be largely of the opposite sequence, again emphasizes the special position of 5-methylcytidylic acid in this nucleic acid.

It is a pleasure to acknowledge the technical assistance, at various times, of James F. Koerner, Jostein Vadla, Kenneth Lunan, and Bennie Friesen.

SUMMARY

The ratios of the sequential isomers of those dinucleotides composed of unlike mononucleotides have been determined in the digest of calf thymus DNA by pancreatic DNase. Among those dinucleotides containing a purine and a pyrimidine nucleotide, there is a great excess of the isomer with the sequence purine nucleotide-pyrimidine nucleotide, with the striking exception of the dinucleotide containing deoxycytidylic and deoxyguanylic acids.

BIBLIOGRAPHY

1. Sinsheimer, R. L., *J. Biol. Chem.*, **208,** 445 (1954).
2. Sinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198,** 293 (1952).
3. Hurst, R. O., and Butler, G. C., *J. Biol. Chem.*, **193,** 91 (1951).
4. Michelson, A. M., and Todd, A. R., *J. Chem. Soc.*, 34 (1954).

[2] The amounts of MC obtained have been too small to permit an analysis for the ratio of sequential isomers.

5. Kutscher, W., and Wörner, A., *Z. physiol. Chem.*, **239,** 109 (1936).
6. Gulland, J. M., and Jackson, E. M., *Biochem. J.*, **32,** 590 (1938).
7. Sinsheimer, R. L., and Koerner, J. F., *Science*, **114,** 42 (1951).
8. Cohn, W. E., *J. Am. Chem. Soc.*, **72,** 1471 (1950).
9. Smith, J. D., and Markham, R., *Nature*, **170,** 120 (1952).

(*Fro

Rec
of me
has b
rat liv
hormo
hepati
of the
is ext
and re
these
enzym
ried o
zymes
ring *

Liv
decap
moge
Potte
the h
cubat
0.1 m
at 37°
incub
tratio
then
with
times
discar
ture

* T
tion,
United

XUM

METABOLISM OF CORTISONE BY RAT LIVER HOMOGENATE*

By ALBERT B. EISENSTEIN

(From the Nutrition Laboratory, Department of Preventive Medicine, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, January 4, 1955)

Recent investigations have demonstrated that the liver is the major site of metabolism of the adrenocortical steroids, including cortisone. This has been shown by various techniques including perfusion of the intact rat liver and incubation of liver slices and homogenates with adrenocortical hormones. Several alterations in the steroid molecule occur during the hepatic metabolism of cortisone. The most important change is reduction of the conjugated, unsaturated system of ring A (1, 2). In addition there is extensive degradation of the 17,21-dihydroxy-20-ketone side chain (2) and reduction of the ketone at C-11 to a hydroxyl group (3, 4). Although these changes have been considered to be enzymatic, little is known of the enzyme systems concerned. The investigations reported herein were carried out in an attempt to determine some of the characteristics of the enzymes concerned with reduction of the conjugated, unsaturated bonds in ring A and degradation of the dihydroxyketone side chain of cortisone.

Procedure

Liver tissue was obtained from male Sprague-Dawley rats killed by decapitation. The liver was removed immediately after death and homogenized in cold Krebs' solution buffered with phosphate to pH 7.4. A Potter-Elvehjem glass homogenizer was used. An aliquot of 4.5 ml. of the homogenate, equivalent to 0.50 gm. of liver, was placed in each incubation flask in addition to 300 γ of cortisone (free alcohol) dissolved in 0.1 ml. of absolute ethyl alcohol. The flasks were stoppered and incubated at 37° for 1 hour. On completion of incubation, acetone was added to the incubation mixture in amount sufficient to attain a final acetone concentration of 70 per cent. The mixture was allowed to stand overnight and then was filtered. The flask and tissue residue were washed repeatedly with hot 70 per cent acetone. The acetone solution was extracted three times with 0.3 volume of petroleum ether and the petroleum ether fraction discarded. The acetone was removed by distillation *in vacuo* at a temperature of 45°. The remaining aqueous solution was extracted three times

* This investigation was supported by research grants from the Nutrition Foundation, New York, and the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, A-442R.

XUM

with 2.5 volumes of ethylene dichloride, the latter fraction dried over sodium sulfate, and the ethylene dichloride removed under vacuum. The residue containing the steroids was dissolved in chloroform and adsorbed on a Florisil column. Various steroid fractions were collected by the chromatographic method of Nelson and Samuels (5). Aliquots of the fraction containing cortisone were taken to dryness under a stream of nitrogen and dissolved in methyl alcohol. The methyl alcohol solution was used for determination of the ultraviolet absorption spectrum which was measured on a Beckman model DU spectrophotometer in the range of 220 to 260 mμ. Maximal absorption at 238 mμ was used for the quantitative determination of the unsaturated ketone structure in ring A. Measurement of the conjugated, unsaturated bonds was achieved also by utilizing the sulfuric acid-chromogen technique of Zaffaroni (6). The absorption peak of the sulfuric acid derivative of cortisone at 280 to 285 mμ is due to the conjugated, unsaturated system (2). The quantity of steroid with the 17,21-dihydroxy-20-ketone side chain was determined by the sulfuric acid-phenylhydrazine method of Porter and Silber (7).

In each experiment, unincubated flasks containing cortisone and liver homogenate were treated by the procedures described above. The values obtained were used as controls and served as a basis for calculating the rate of disappearance of the conjugated, unsaturated bonds of ring A and degradation of the side chain in the incubated flasks. Also included in each experiment were flasks which contained liver homogenate but no cortisone. These gave a small absorption at the wave-lengths used for determining the amount of steroid present, and the values were, therefore, subtracted from those obtained from vessels containing both cortisone and liver homogenate. The recovery of cortisone was determined in each experiment and ranged from 75 to 95 per cent.

In an effort to find factors which would influence the rate of metabolism of cortisone by liver homogenate, various substances were added to the incubation mixture. These included sodium citrate, isocitric acid, DPN,[1] sodium pyruvate, sodium succinate, α-ketoglutaric acid, adenosine triphosphate, adenosine-5-phosphoric acid, malic acid, and Versene. Whenever a particular compound was added, the experiment was further controlled by incubating this substance with liver homogenate and analyzing the mixture by the method described previously. Corrections were made by subtracting these values from those in which the particular compound was incubated with liver tissue and cortisone.

Results

Incubation of rat liver homogenate and cortisone for 1 hour in the physiologic medium used resulted in an average loss of 20 per cent of the con-

[1] DPN = diphosphopyridine nucleotide.

jugated unsaturated bonds of ring A and average degradation of 26 per cent of the 17,21-dihydroxyketone side chain.

TABLE I

Effect of Citrate on Hepatic Metabolism of Cortisone

	Ultraviolet spectroscopy		Sulfuric acid color reaction		Porter-Silber reaction	
	Average optical density at 238 mμ	Per cent loss of conjugated unsaturated bonds	Average optical density at 280 mμ	Per cent loss of conjugated unsaturated bonds	Average optical density at 410 mμ	Per cent loss of dihydroxyketone structure
A. Unincubated controls	0.185		0.290		0.223	
B. Incubated 1 hr. in phosphate buffer	0.149*	19.5	0.220*	24.2	0.134*	39.9
C. Incubated 1 hr. in phosphate buffer + 0.01 M citrate	0.096†	48.1	0.155†	46.6	0.063†	71.7
D. Incubated 1 hr. in phosphate buffer + 0.001 M citrate	0.096†‡	48.1	0.159†‡	45.2	0.067†	70.0

* Difference from control value (A) significant, $P = <0.01$.

† Difference from incubated control (B) significant, $P = <0.01$.

‡ Duplicate samples; all others triplicate. All samples obtained from one rat liver.

TABLE II

Effect of Isocitrate on Hepatic Metabolism of Cortisone

	Ultraviolet spectroscopy		Sulfuric acid color reaction		Porter-Silber reaction	
	Average optical density at 238 mμ	Per cent loss of conjugated unsaturated bonds	Average optical density at 280 mμ	Per cent loss of conjugated unsaturated bonds	Average optical density at 410 mμ	Per cent loss of dihydroxyketone structure
A. Unincubated controls	0.195		0.338*		0.252	
B. Incubated 1 hr. in phosphate buffer	0.168†	13.9	0.276‡	18.3	0.196‡	22.3
C. Incubated 1 hr. in phosphate buffer + 0.01 M isocitrate	0.135§	30.8	0.214§	36.7	0.124§	50.8

* Duplicate samples; all others triplicate. All samples obtained from one rat liver.

† Difference from control value (A) significant, $P = <0.05$.

‡ Difference from control value (A) significant, $P = <0.01$.

§ Difference from incubated sample (B) significant, $P = <0.01$.

Table I demonstrates the effect of addition of citrate to attain a 0.01 M or 0.001 M concentration in the incubation mixture. The results are compared with those obtained after incubation of cortisone and liver homogenate in the buffered solution. It is apparent that the loss of conjugated, unsaturated bonds was approximately doubled after addition of either 0.01 or 0.001 M citrate when measured by ultraviolet spectroscopy and the sulfuric acid technique of Zaffaroni. The rate of degradation of the dihydroxyketone side chain was increased almost 2-fold as determined by the Porter-Silber technique.

TABLE III

Effect of DPN on Hepatic Metabolism of Cortisone

	Ultraviolet spectroscopy		Porter-Silber reaction	
	Average optical density at 238 mμ	Per cent loss of conjugated unsaturated bonds	Average optical density at 410 mμ	Per cent loss of dihydroxyketone structure
A. Unincubated controls	0.217*		0.297*	
B. Incubated 1 hr. in phosphate buffer	0.161*†	25.8	0.183*‡	38.4
C. Incubated 1 hr. in phosphate buffer + DPN and niacinamide	0.128§	41.0	0.151§	49.2
D. Incubated 1 hr. in phosphate buffer + DPN	0.155‖	28.7	0.171‖	42.4

* Duplicate samples; all others triplicate. All samples obtained from one rat liver.

† Difference from control value (A) significant, $P = <0.05$.

‡ Difference from control value (A) significant, $P = <0.01$.

§ Difference from incubated control (B) significant, $P = <0.05$.

‖ Difference from incubated control (B) not significant.

When isocitric acid was added to the incubation mixture in 0.01 M concentration, there was also a marked increase in the rate of destruction of the conjugated, unsaturated bonds and degradation of the dihydroxyketone side chain. The results of this experiment are presented in Table II.

In Table III, the results following addition of 24 μmoles of DPN and 0.001 M niacinamide to the incubation mixture are compared with those in which neither DPN nor niacinamide was added and also with results obtained after adding DPN but not niacinamide. The addition of both DPN and niacinamide considerably accelerated the rate of saturation of the α,β-unsaturated ketone, but caused a lesser increase in degradation of

the side chain. It is also apparent that addition of DPN without niacinamide did not alter the rate of either of these reactions.

The addition of α-ketoglutaric acid to the incubation mixture of liver homogenate and cortisone in 0.01 M concentration resulted in almost complete inhibition of the inactivation of cortisone (see Table IV).

None of the other substances added to the system in an effort to affect the rate of reaction had any influence on the metabolism of cortisone.

TABLE IV

Effect of α-Ketoglutarate on Hepatic Metabolism of Cortisone

	Ultraviolet spectroscopy		Sulfuric acid color reaction		Porter-Silber reaction	
	Average optical density at 238 mμ*	Per cent loss of conjugated unsaturated bonds	Average optical density at 280 mμ*	Per cent loss of conjugated unsaturated bonds	Average optical density at 410 mμ*	Per cent loss of dihydroxyketone structure
A. Unincubated controls	0.181		0.298		0.218	
B. Incubated 1 hr. in phosphate buffer	0.145†	19.9	0.244†	18.1	0.165†	24.3
C. Incubated 1 hr. in phosphate buffer + 0.01 M α-ketoglutarate	0.178‡	1.7	0.279‡	6.4	0.200‡	8.3

* Triplicate samples obtained from one rat liver.

† Difference from control values (A) significant, $P = <0.01$.

‡ Difference from incubated control (B) significant, $P = <0.01$.

DISCUSSION

Citrate has been shown to accelerate the hepatic metabolism of progesterone and testosterone (8, 9). It has been suggested that the effect of citrate on the metabolism of progesterone is due to the removal of metallic ions which inhibit reduction of the conjugated, unsaturated bonds of ring A (8). Other evidence indicates that citrate functions as a cofactor for a different enzyme system in the saturation of the conjugated, unsaturated bonds of testosterone, since metal-binding agents do not increase the metabolism of this hormone (8). The results of this investigation have demonstrated that citrate accelerates the hepatic metabolism of cortisone. The addition of citrate increases the rate of disappearance of the α,β-unsaturated ketone structure and degradation of the side chain. Citrate does not function in the same manner in this enzyme system as in the metabolism of progesterone, since the addition of a chelating agent, Versene, did not influence the rate of metabolism of cortisone. It is also apparent that citrate does not function solely as an intermediate of the Krebs cycle,

since the addition of succinate and malate did not increase the inactivation of this steroid. It is, therefore, believed that citrate may function as a cofactor important to or necessary for the metabolism of cortisone by liver tissue.

DPN has previously been demonstrated to increase hepatic metabolism of testosterone and accelerates the conversion of this androgen to a 17-ketosteroid (9). In this study, the addition of DPN and niacinamide facilitated the reduction of the unsaturated, conjugated bonds, but had less effect on degradation of the dihydroxyketone side chain. The addition of niacinamide as well as DPN to the incubation mixture was important, since DPN alone did not alter the rate of these reactions. It is thought that niacinamide inhibits destruction of DPN by a specific enzyme present in hepatic tissue. The formation of 17-ketosteroids was not determined in this experiment, but it seems unlikely that appreciable amounts of this type of steroid were formed, since DPN had relatively little effect on degradation of the side chain of cortisone. The mechanism by which DPN accelerates reduction of the conjugated, unsaturated bonds is not apparent.

The addition of α-ketoglutarate to the incubation mixture resulted in an almost complete inhibition of the metabolism of cortisone by liver homogenate. It is known that α-ketoglutarate requires DPN as a hydrogen acceptor before it can be converted to succinate. It is suggested that the inhibition of steroid metabolism following addition of this dicarboxylic acid may result from competition between it and the steroid for the DPN in the system.

SUMMARY

1. It has been shown that the α,β-unsaturated ketone structure and the 17,21-dihydroxy-20-ketone side chain of cortisone disappear on incubation with liver homogenate.

2. The metabolism of cortisone is accelerated by citrate and isocitrate, which increase the rate of disappearance of both the conjugated, unsaturated bonds of ring A and the side chain.

3. DPN accelerates hepatic metabolism of cortisone by increasing reduction of the α,β-unsaturated ketone, but has less effect on degradation of the side chain.

4. The metabolism of cortisone by rat liver homogenate is almost completely inhibited by α-ketoglutarate.

BIBLIOGRAPHY

1. Caspi, E. Y., Levy, H., and Hechter, O. M., *Arch. Biochem. and Biophys.*, **45**, 169 (1953).
2. Schneider, J. J., and Horstmann, P. M., *J. Biol. Chem.*, **196**, 629 (1952).
3. Eisenstein, A. B., *Proc. Soc. Exp. Biol. and Med.*, **83**, 27 (1953).

4. Fish, C. A., Hayano, M., and Pincus, G., *Arch. Biochem. and Biophys.*, **42**, 480 (1953).
5. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metabolism*, **12**, 519 (1952).
6. Zaffaroni, A., *J. Am. Chem. Soc.*, **72**, 3828 (1950).
7. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, **185**, 201 (1950).
8. Wiswell, J. G., and Samuels, L. T., *J. Biol. Chem.*, **201**, 155 (1953).
9. Levedahl, B. H., and Samuels, L. T., *J. Biol. Chem.*, **186**, 857 (1950).

HYD
C

By

(

The
matte
this c
terium
Little
rates
deute
releva

Erl
bioche
that,
of sel
deute
Erlen
when
porte
genas
lene b
succi
meter
about
substr

It
uptak
burg
The s
obser
terios

The

* O
July, 1
† A
anami

XUM

HYDROGEN ISOTOPE EFFECT IN THE OXIDATION IN VIVO OF METHIONINE LABELED IN THE METHYL GROUP

By JULIAN R. RACHELE, EDWARD J. KUCHINSKAS, F. HOWARD KRATZER,* AND VINCENT DU VIGNEAUD†

(From the Department of Biochemistry, Cornell University Medical College, New York, New York)

(Received for publication, January 18, 1955)

The retarding effect of deuterium on biological processes has been a matter of interest to many investigators. However, the early studies in this connection were concerned mainly with the biological effects of deuterium when it was present in excess amounts in the water of the medium. Little work appears to have been done on the effect of deuterium on the rates of biological reactions in which carbon-hydrogen bonds labeled with deuterium are broken during necessary metabolic changes. In 1936, two relevant reports appeared in the literature.

Erlenmeyer, Schoenauer, and Süllmann (1) studied the chemical and biochemical dehydrogenation of α,α'-dideuteriosuccinic acid. They found that, when hydrogen is removed from the deuteriosuccinic acid by the use of selenium dioxide, the fumaric acid formed had about 10 per cent more deuterium in the α,α' positions than the initial compound. In other words, Erlenmeyer *et al.* had demonstrated an isotope effect on a chemical reaction when deuterium is present in a reactant. In biochemical experiments reported in the same paper, in which a crude preparation of succinic dehydrogenase from beef muscle was used, the hydrogen was transferred to methylene blue in a Thunberg tube in about 30 per cent less time from normal succinic than from the deuteriosuccinic acid, while, in the Warburg manometer, the rate of oxygen consumption with the same enzyme system was about 40 per cent less with the deuteriosuccinic acid than with the normal substrate.

It was shown by Sonderhoff and Thomas (2) that the rate of oxygen uptake by yeast suspensions in the presence of trideuterioacetate in Warburg vessels was about 80 per cent of that in the presence of normal acetate. The same degree of retardation in the rate of oxygen consumption was observed by these workers in the dehydrogenation of $\alpha,\alpha',\beta,\beta'$-tetradeuteriosuccinate catalyzed by an enzyme preparation from horse heart.

The kinetics of the enzymatic oxidation of deuterated succinic acid was

* On leave from the University of California at Davis, January, 1953, through July, 1953.

† Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this investigation.

XUM

studied in greater detail only recently by Thorn (3). Using more active preparations of succinic dehydrogenase in order to minimize the exchange effects observed in the earlier studies, Thorn was able to confirm and extend the findings of previous experiments by the Erlenmeyer and Sonderhoff teams. The difference in activation energies between normal succinic acid and tetradeuteriosuccinic acid is given by Thorn as about 1500 calories, with the higher activation energy being associated with the deuterated substrate. This difference in activation energy is of the same order of magnitude as the difference between the residual vibrational energies of the C—H and C—D bonds at the absolute zero temperature that can be calculated from the vibrational frequencies of the respective bonds (4).

In the course of investigating the utilization of the methyl groups of various precursors and the synthesis *in vivo* of labile methyl groups, use has been made of C^{14}-labeled and of C^{14}- and deuterium-labeled compounds. In order to determine whether methanol entered into transmethylation reactions of the body, du Vigneaud and Verly (5) administered C^{14}-labeled methanol to rats and demonstrated the incorporation of the carbon of methanol into the methyl groups of tissue choline.

In a further study of 1-carbon compounds involved in the biosynthesis of labile methyl groups (6), methanol, doubly labeled with C^{14} and deuterium in the methyl group, was given to rats. From the change in ratio of deuterium to C^{14} in the methyl groups of the tissue choline from that in the administered methanol, it was concluded that methanol was utilized in part in the body of the rat by a process of oxidation with subsequent reduction to newly formed labile methyl groups.

With a view towards the use of tritium as a sensitive tracer of hydrogen in reactions in which carbon-hydrogen bonds are broken and reformed, the neogenesis of labile methyl groups from methanol was again studied in the rat. In two separate experiments, preparations of methanol, labeled with different numbers of deuterium atoms and also with C^{14} and tritium (7, 8), were administered to rats, and the methyl groups of tissue choline were analyzed for the relative degree of incorporation of deuterium and tritium. The greater tritium to deuterium ratio in the methyl groups of choline, as compared to that in the administered methanol, indicated a larger retention of tritium than of deuterium by the carbon of methanol during its incorporation into choline methyl, again demonstrating the concept that, in general, chemical bonds involving the lighter isotopes of elements are more reactive.[1]

[1] Dr. Cosmo G. Mackenzie of the University of Colorado School of Medicine has informed us in a private communication that he and Mr. Robert Abeles have studied recently the oxidation of sarcosine, with a mitochondrial preparation in a closed system in which the reactants and products were quantitatively accounted for. The sarcosine, labeled in the methyl group, was a mixture of the $C^{14}H_3$- and CD_3-contain-

The biological selection among the hydrogen isotopes, then, poses a problem when the metabolic pathway of a substrate is to be studied by double labeling, for example, with C^{14} and deuterium. Double labeling of compounds has usually been accomplished by the admixture of singly labeled isotopic species, a procedure that can be referred to as intermolecular double labeling. Since protium bonds do react in general more rapidly than deuterium or tritium bonds, then a disproportionation can occur between C^{14} and deuterium from substrate to product, not only because of a particular metabolic reaction but also because of an isotope effect between protium and the heavier isotopes of hydrogen. The extent of the isotope effect is, as has been shown by Bigeleisen (9), a function of the fraction of a substrate that has been converted in a particular chemical reaction. The dependence of the isotope effect on the percentage of conversion of an isotopic substrate is clearly indicated in studies on the oxidation of 2-deuteriopropanol-2 by Westheimer and Nicolaides (10), of tritium-labeled benzyl alcohol by Hodnett and Kaplan (11), and of tritium-labeled ethanol by Kaplan (12). It follows, then, that the degree of disproportionation between C^{14} and deuterium will be similarly affected by the degree of completion of a chemical reaction.[2] Since the biochemical reactions of a substrate *in vivo*, as well as *in vitro* with isolated tissues, can be manifold, disproportionation of the C^{14}:D ratio due to isotope effects will be difficult to evaluate.

The present report deals with the direct demonstration of the retarding effect of deuterium on the rate of appearance of the deuterium-bearing carbon of a metabolite in the respiratory carbon dioxide of the intact rat. For this purpose it was necessary to prepare a metabolite intramolecularly doubly labeled in such a way that the isotopic labeling atoms of carbon and of hydrogen are bonded to each other.

The compound chosen for this purpose was methionine which, when labeled with C^{14} and deuterium in the methyl group, has been investigated in this laboratory in transmethylation (13) and in studies on the oxidation of the methyl group (14, 15) following administration in the rat. In a rate of oxidation experiment (15), the methionine labeled in the methyl group with C^{14} was administered as a single dose to animals either *per os* at a level of 1.2 per cent in a purified diet, or parenterally at the same level with the simultaneous ingestion of a 2 gm. meal. In the first 6 hour period following the dose, 27 per cent of the C^{14}-methyl group was oxidized to CO_2. It was intended originally, for the present study, to administer to any individual

ing species. The formaldehyde isolated from the reaction mixture showed an increase in the C^{14} specific activity that indicates a large degree of selection between protium and deuterium in the oxidation of the methyl group.

[2] A discussion of the various cases of double labeling of a carbon atom and its bonded hydrogen atoms was presented in a previous publication (8).

animal a single dose of methionine, triply labeled in the methyl group by admixture of one isotopic species containing $C^{14}H_3$-methyl groups with another isotopic species containing $C^{13}D_3$-methyl groups, and to follow in the rat the disproportionation, if any, in the C^{14}:C^{13} ratio of the administered methionine when the carbon labels appeared in the respiratory CO_2. This procedure would have eliminated, as in the experiments with triply labeled methanol containing C^{14}, D, and T, the uncertainties introduced by the biological variations accompanying separate employment of protium- and deuterium-bonded species of labeled methionine in different animals. However, calculations showed that, on the basis of the earlier rate study with C^{14}-methyl-labeled methionine (15), even at the time when the rate of appearance of the label in the expired CO_2 is maximal the atom per cent excess of C^{13} would be too low to be of much significance in the presence of the large natural abundance of C^{13}. It was decided, therefore, to measure the appearance in the expired CO_2 of radioactive carbon following the administration of methionine containing either the singly labeled $C^{14}H_3$-methyl group *or* the intramolecularly doubly labeled $C^{14}D_3$-methyl group in separate single doses in consecutive experiments in the same animal.

EXPERIMENTAL

Preparation of Labeled Compounds

$C^{14}H_3$-Methionine—L-Methionine, labeled with C^{14} in the methyl group, was prepared according to the method of Melville, Rachele, and Keller (16), starting with C^{14}-methanol. The specific activity of the $C^{14}H_3$-methionine was determined as 4.22×10^5 c.p.m. per mg. of compound. Nitrogen (Kjeldahl) found, 9.3; calculated,[3] 9.4.

$C^{14}D_3$-Methionine—Intramolecularly doubly labeled methanol was prepared by an adaptation of the procedures described by Nystrom, Yanko, and Brown (17), and by Cox, Turner, and Warne (18). Radioactive carbon dioxide[4] was generated from $BaC^{14}O_3$ with the addition of phosphoric acid, and the gas was carried in a stream of nitrogen through a magnesium perchlorate drying tube into a solution of lithium aluminum deuteride[5] dissolved in diethyl carbitol. By this process, the carbon dioxide was reduced to methanol, doubly labeled with deuterium and C^{14} in the same methyl group. *n*-Butyl carbitol was added to the reaction mixture to decompose the lithium aluminum methoxy complex which had formed and to release the methanol. Non-isotopic methanol was added as carrier, and

[3] The calculated value does not account for the increased molecular weight due to C^{14} in the molecule.

[4] The radioactive barium carbonate was obtained from the Oak Ridge National Laboratory on allocation by the United States Atomic Energy Commission.

[5] The lithium aluminum deuteride was obtained from Metal Hydrides Incorporated on allocation by the United States Atomic Energy Commission.

the mixture of methanols was flushed out of the reaction vessel into a trap immersed in a dry ice-ethanol bath. Doubly labeled methyl iodide was then prepared by passing the methanol as a vapor through a refluxing solution of deuterium iodide made from P, I_2, and D_2O. The methyl iodide was incorporated into L-methionine by the procedure used above in the preparation of the $C^{14}H_3$-methionine. Determination of the C^{14} content of the methionine gave 1.45×10^5 c.p.m. per mg. of compound. Analysis for deuterium gave 1.63 atom per cent excess in the compound which corresponded to 6.00 atom per cent excess deuterium in the methyl group. Nitrogen (Kjeldahl) found, 8.8; calculated,[6] 9.4.

Administration of Labeled Methionines and Assay of Respiratory CO_2— The apparatus employed throughout the experiment was essentially that described in earlier experiments from this Laboratory (14). The animals were placed in the metabolism chamber about 16 hours prior to the injection of the labeled compounds, and their respiratory carbon dioxide was collected in two scrubbers, each containing 400 ml. of 2.5 N NaOH. During the course of the preinjection and experimental periods, free access was permitted to water and to a casein diet ((19), without added cystine).

At the commencement of the experimental period, each animal received an intraperitoneal injection of 0.16 mmole of a labeled methionine preparation which was dissolved in 1 ml. of water. The animal was placed immediately in the metabolism chamber and collections of the respiratory carbon dioxide were begun. The expired carbon dioxide was collected at hourly intervals for at least 7 hours. The alkali from each hourly interval was diluted to 1 liter and an aliquot removed for the determination of the C^{14} activity. After correcting each sample for background and self-absorption and applying the appropriate dilution factor, the radioactivity of the respiratory carbon dioxide was compared to the radioactivity of the injected dose of methionine. If the animal had at any time previously received any C^{14}, the rate of appearance of $C^{14}O_2$ during the preinjection period was used as the control value and was subtracted from the observed rate during the experimental period. The correction per hour due to the preinjection radioactivity was approximately 0.06 to 0.07 per cent of the dose. This correction is small compared to the rates of $C^{14}O_2$ production observed during the experimental period. The total percentage of the radioactivity in the dose, which had appeared in the respiratory carbon dioxide at hourly intervals, is given in Table I.

DISCUSSION

If an isotope effect is present in the catabolism of the methyl group of methionine due to the linkage of the C^{14} atoms therein to either protium or

[6] The contribution of the deuterium and C^{14} content of the molecule to the molecular weight is negligible.

deuterium, then the percentage of the total administered radioactive carbon expired over a period of several hours should be less when the C^{14} atoms

TABLE I

Percentage of Radioactivity of Administered Methionine Appearing in Respiratory Carbon Dioxide

Rat No.	Weight	Methyl group	Time after injection of labeled methionine						
			1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.
	gm.								
549	379	$C^{14}H_3$—	7.1	13.5	16.4	17.8	18.6	19.3	19.8
549	418	$C^{14}D_3$—	4.7	9.9	12.8	14.3	15.3	16.0	16.6
550	328	$C^{14}H_3$—	7.3	14.0	17.0	18.3	19.1	19.6	20.1
550	361	$C^{14}D_3$—	4.2	9.3	12.0	13.5	14.4	15.0	15.6

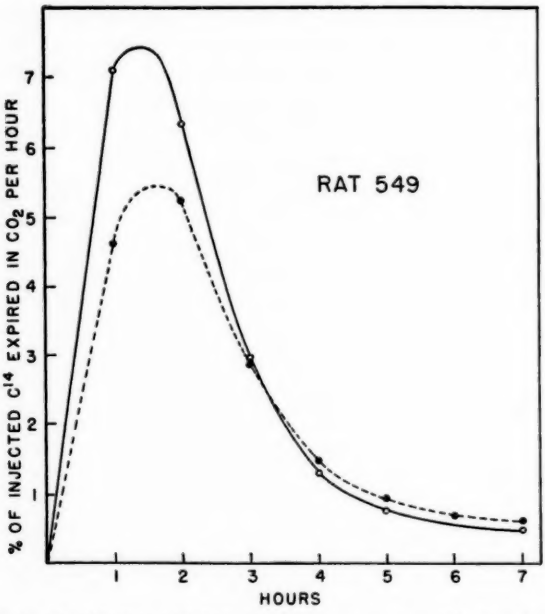


FIG. 1. Rate of oxidation of labeled methyl group of methionine in Rat 549; ○, $C^{14}H_3$ group; ●, $C^{14}D_3$ group.

are bonded to deuterium. The results listed in Table I show this to be so. For example, at the 2nd hour after the administration of the $C^{14}D_3$-methionine, the percentages of the total C^{14} dose expired by two rats as $C^{14}O_2$ were

73 and 66 per cent, respectively, of the percentages expired after giving $C^{14}H_3$-methionine. As can be noted in Table I, this difference decreased with time. This diminution is to be expected as the administered mixture becomes more completely metabolized. Over a 7 hour period following administration, the $C^{14}D_3$ group is oxidized at about 60 to 80 per cent of the level at which the $C^{14}H_3$ group is oxidized. It is interesting to note that the degree of retardation of the metabolic processes observed in these

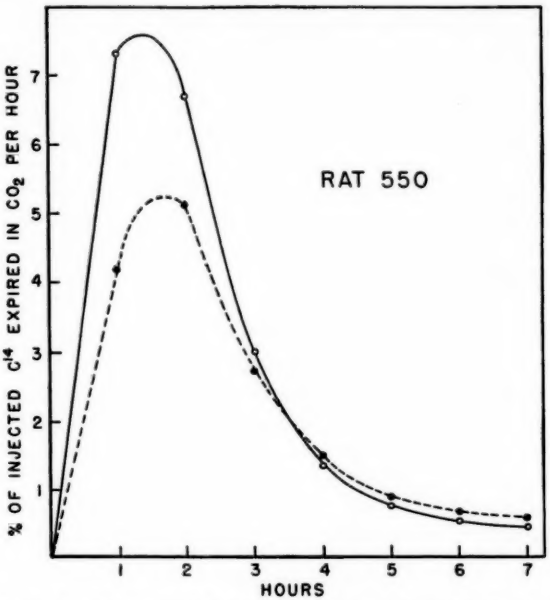


FIG. 2. Rate of oxidation of labeled methyl group of methionine in Rat 550; ○, $C^{14}H_3$ group; ●, $C^{14}D_3$ group.

experiments in the intact rat is of the same order of magnitude as that observed by other workers with isolated enzyme systems (1–3).

If the hourly appearance of the administered C^{14} in the expired CO_2 expressed as percentage of the injected dose is plotted as a function of time, the curves shown in Figs. 1 and 2 are obtained. It will be noted that the rate of excretion of $C^{14}O_2$ during the first phase (15) of the metabolism of the $C^{14}D_3$-methyl group of the labeled methionine is less than that from the $C^{14}H_3$-methyl group up to some time between the 3rd and 4th hours after injection, after which time the rates are reversed. Thus, during the phase of attaining a steady state (15), slightly more $C^{14}O_2$ per hour is being

excreted following the administration of the $C^{14}D_3$-methionine than after the $C^{14}H_3$-methionine. The observed differences in the rates of oxidation of the methyl group of $C^{14}H_3$-methionine and $C^{14}D_3$-methionine in both phases is consistent with an isotope effect due to the bonding of C^{14} to protium on the one hand and to deuterium on the other. The slower rate of metabolism of the $C^{14}D_3$ group during the first phase as compared with the $C^{14}H_3$ species permits a higher concentration of the deuterated C^{14} substrate or intermediates to remain for metabolism or utilization in the later phase.

The results of this experiment, together with the results in the earlier reports on methyl synthesis from C^{14}-, deuterium-, and tritium-containing methanol (7, 8), clearly indicate that double labeling of a single carbon unit in a metabolite must be of the type that minimizes as much as possible any ambiguity due to isotope effects, primarily of hydrogen.

The authors wish to thank Mr. Robert J. Brotherton for his valuable assistance in the determination of the radioactivity of the respiratory carbon dioxide and Mr. Joseph Albert for the microanalyses performed in connection with this experiment.

SUMMARY

A hydrogen isotope effect has been demonstrated by a comparison *in vivo* of the oxidation of labeled methionine preparations in which C^{14} atoms in the methyl group are bonded either to protium atoms or to deuterium atoms. The methyl groups in which the C^{14} is bonded to deuterium were oxidized at a rate from 60 to 80 per cent of the rate of oxidation of methyl groups containing the C^{14} bonded to protium. The need for appropriate double labeling of single carbon units for metabolic studies is emphasized.

BIBLIOGRAPHY

1. Erlenmeyer, H., Schoenauer, W., and Süllmann, H., *Helv. chim. acta*, **19,** 1376 (1936).
2. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530,** 195 (1937).
3. Thorn, M. B., *Biochem. J.*, **49,** 602 (1951).
4. Eyring, H., and Sherman, A., *J. Chem. Phys.*, **1,** 345 (1933).
5. du Vigneaud, V., and Verly, W. G., *J. Am. Chem. Soc.*, **72,** 1049 (1950).
6. du Vigneaud, V., Verly, W. G. L., Wilson, J. E., Rachele, J. R., Ressler, C., and Kinney, J. M., *J. Am. Chem. Soc.*, **73,** 2782 (1951).
7. Verly, W. G., Rachele, J. R., du Vigneaud, V., Eidinoff, M. L., and Knoll, J. E., *J. Am. Chem. Soc.*, **74,** 5941 (1952).
8. Rachele, J. R., Kuchinskas, E. J., Knoll, J. E., and Eidinoff, M. L., *J. Am. Chem. Soc.*, **76,** 4342 (1954).
9. Bigeleisen, J., *Science*, **110,** 14 (1949).
10. Westheimer, F. H., and Nicolaides, N., *J. Am. Chem. Soc.*, **71,** 25 (1949).
11. Hodnett, E. M., and Kaplan, L., Abstracts, American Chemical Society, 125th meeting, Kansas City, 26N (1954).

12. Kaplan, L., *J. Am. Chem. Soc.*, **76,** 4645 (1954).
13. Keller, E. B., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **177,** 733 (1949).
14. Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., and du Vigneaud, V., *J. Biol. Chem.*, **180,** 99 (1949).
15. Mackenzie, C. G., Rachele, J. R., Cross, N., Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **183,** 617 (1950).
16. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169,** 419 (1947).
17. Nystrom, R. F., Yanko, W. H., and Brown, W. G., *J. Am. Chem. Soc.*, **70,** 441 (1948).
18. Cox, J. D., Turner, H. S., and Warne, R. J., *J. Chem. Soc.*, 3167 (1950).
19. du Vigneaud, V., Verly, W. G., and Wilson, J. E., *J. Am. Chem. Soc.*, **72,** 2819 (1950).

THE MECHANISM OF PORPHYRIN FORMATION

FURTHER EVIDENCE ON THE RELATIONSHIP OF THE CITRIC ACID CYCLE AND PORPHYRIN FORMATION*

By JOHN C. WRISTON, JR.,† LEON LACK,‡ AND DAVID SHEMIN

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York)

(Received for publication, January 10, 1955)

We have previously demonstrated the intimate relationship of the citric acid cycle and porphyrin formation (2, 3). The compound common to both these systems is "active" succinate, which is produced in the citric acid cycle and utilized, after condensation with glycine, for all the carbon atoms of the porphyrin except those arising from the α-carbon atom of glycine. In order to document this relationship further we have more recently studied the utilization of α-ketoglutarate-5-C^{14}, α-ketoglutarate-1,2-C^{14}, and citrate-1,5-C^{14} (1) for porphyrin formation. Confirmation was also obtained in experiments in which the utilization of both methyl- and carboxyl-labeled acetate for porphyrin formation was studied in the presence and absence of malonate (1).

The participation of the citric acid cycle in porphyrin synthesis is indicated in Fig. 1. Not only should C^{14}-labeled members of the cycle produce C^{14}-labeled protoporphyrin, but one should be able to predict, as we have done previously (2, 3), which carbon atoms of the porphyrin are radioactive or arise from particular carbon atoms of the substrate. Therefore, if members of the cycle other than those studied previously produce labeled protoporphyrin and the predicted carbon atoms are radioactive, then the formulation in Fig. 1 would be further supported.

It may be also well to point out that this pathway of porphyrin synthesis may be considered, from an experimental point of view, as a natural trap-

* This work was supported by grants from the National Institutes of Health, United States Public Health Service (No. RG-1128), from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and from the Rockefeller Foundation. The results were reported at the meeting of the American Society of Biological Chemists at Chicago, April, 1953 (1). This report is part of the dissertations submitted by Leon Lack and by John C. Wriston, Jr., in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

† Fellow of the Life Insurance Medical Research Fund, 1951–53. Present address, Department of Biochemistry, University of Colorado, Denver, Colorado.

‡ Present address, Department of Biochemistry, Duke University, Durham, North Carolina.

XUM

ping mechanism for the "active" succinate, and thus certain aspects of the citric acid cycle can be studied by investigation of the mechanism of porphyrin synthesis.

We have found that, whereas succinate can readily penetrate the intact duck red blood cell, α-ketoglutarate and citrate do not. Therefore, in the experiments reported here, hemolyzed preparations of duck red blood cells were used. These preparations, as shown earlier, are quite capable of synthesizing heme (3, 4).

It was found that incubation of this biological preparation with α-ketoglutarate-1,2-C^{14}, α-ketoglutarate-5-C^{14}, and with citrate-1,5-C^{14} resulted in the production of radioactive hemin.

α-Ketoglutarate-5-C^{14} and citrate-1,5-C^{14} should directly label the carboxyl groups of protoporphyrin (*C*-10, *D*-10) (Fig. 2) as we have previously

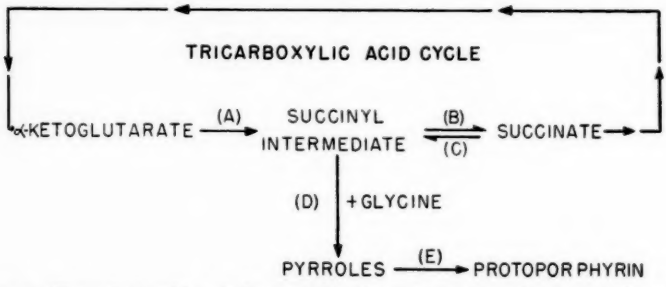


FIG. 1. The relationship of the tricarboxylic acid cycle and protoporphyrin formation.

formulated (2, 3). In the citric acid cycle, citrate-1,5-C^{14} should produce α-ketoglutarate-1,5-C^{14} exclusively, and consequently both substrates studied (citrate-1,5-C^{14} and α-ketoglutarate-5-C^{14}) should be equivalent in their biological utilization pattern for porphyrin formation, since the α-carboxyl group of α-ketoglutarate is given off in its conversion to "active" succinate. Further, since carboxyl-labeled acetate produces α-ketoglutarate-1,5-C^{14}, the pattern of labeling in the protoporphyrin synthesized from citrate-1,5-C^{14} and α-ketoglutarate-5-C^{14} should be the same as was previously found in protoporphyrin synthesized from carboxyl-labeled acetate (2). In reference to the labeling pattern of the porphyrin synthesized from these substrates another point must be considered. As we have previously proposed (2) and experimentally demonstrated (3), "active" succinate also arises from succinate (Reaction C, Fig. 1). Therefore, since the succinate behaves as a symmetrical molecule, not only would the carboxyl groups of the porphyrin contain C^{14} from Reactions A and C, but also carbon atoms 3 and 5 (see Figs. 2 and 3) should contain some C^{14} via Reaction C. The

relative radioactivities of carbon atoms 10 and 3 and 5 are a reflection of Reactions A and C (Fig. 1), as are the relative activities of pyrrole rings *A* and *B* and rings *C* and *D*, since the former pyrrole rings bearing no carboxylic acid groups are made radioactive only via Reaction C.

The hemin samples obtained from the experiment in which the hemolyzed duck red blood cell preparations were incubated with citrate-1,5-C^{14} and α-ketoglutarate-5-C^{14} were degraded by the methods peviously published (2, 5), whereby fragments of the porphyrin molecule are isolated and

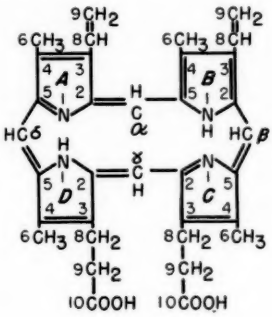


FIG. 2

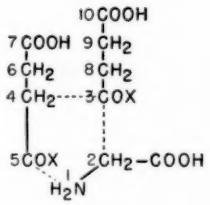


FIG. 3

FIG. 2. Protoporphyrin IX. The above numbering system is the same as that employed previously (2, 5).

FIG. 3. This figure represents the positions of the carbon atoms of "active" succinate and the α-carbon atom of glycine in each pyrrole unit. Since all four pyrrole rings arise from the same precursor pyrrole and since each side of the pyrrole unit arises from the "active" succinate (2, 3) as pictured above, then carbon atom 5 is equivalent to carbon atom 3, carbon atom 4 is equivalent to carbon atom 8, etc. Therefore, it is sufficient to obtain merely the C^{14} distribution pattern of any one fragment of any pyrrole ring; *e.g.*, carbon atoms 6, 4, and 5 of either rings *A* and *B* or of *C* and *D*, or carbon atoms 9, 8, 3, and 2 of either rings *A* and *B* or of *C* and *D*.

individual carbon atoms from known positions in the porphyrin molecule are obtained. It can be seen from Table I that, in agreement with our previous concepts of porphyrin formation, comparable fragments have the same activity (the methylethylmaleimide samples representing rings *A* and *B* and rings *C* and *D*), each half of each pyrrole unit (the pyruvic acid fragment, carbon atoms 6, 4, and 5 and the ketobutyric acid fragment, carbon atoms 9, 8, 3, and 2) has the same radioactivity and, finally, as predicted, only carbon atoms 10, 3, and 5 are radioactive. Carbon atoms 10 are more highly radioactive, whereas carbon atoms 3 and 5 have the same amount of C^{14}.

The formulation of Figs. 1 and 3 requires that α-ketoglutarate-1,2-C^{14} should, on the other hand, give rise to a porphyrin whose labeling pattern is

TABLE I

Distribution of C^{14} Activity in Protoporphyrin Fragments Synthesized from Citric Acid-1,5-C^{14} (0.8 mc. per mmole), α-Ketoglutaric Acid-5-C^{14} (0.06 mc. per mmole), and α-Ketoglutaric Acid-1,2-C^{14} (0.1 mc. per mmole)

The labeled substrates (citric acid, 0.1 mg. per ml. of blood preparation; α-ketoglutaric acid, 0.5 mg. per ml. of blood preparation) and glycine (1 mg. per ml. of blood preparation) were added to the hemolyzed duck red blood cell preparation containing 4 mg. of ferric iron per 100 ml., and the mixture was shaken at room temperature for 20 hours.

Position of fragment in porphyrin and compound analyzed for C^{14} activity	Citric acid-1,5-C^{14}		α-Ketoglutaric acid-5-C^{14}		α-Ketoglutaric acid-1,2-C^{14}	
	Molar activity	Sum of molar activities of fragments	Molar activity	Sum of molar activities of fragments	Molar activity	Sum of molar activities of fragments
	c.p.m.		*c.p.m.*		*c.p.m.*	
Whole porphyrin (hemin)	8600		7400		7600	
Pyrrole rings *A* and *B* (methylethylmaleimide)	1900		1390		3850	
Pyrrole rings *C* and *D* (hematinic acid)	6600		6000		4470	
Sum of pyrrole rings *A–D*		8500		7390		8320
Pyrrole rings *A* and *B*	1900		1390		3850	
Carbons 6, 4, 5 (2,4-dinitrophenylhydrazone of pyruvic acid)	1000		730		2100	
Carbons 9, 8, 3, 2 (2,4-dinitrophenylhydrazone of α-ketobutyric acid)	1000		740		2200	
Sum of activities of carbons 6, 4, 5, 9, 8, 3, 2		2000		1470		4300
Carbon 5 ($BaCO_3$)	800*		580*		1300*	
Carbons 6, 4 (sodium acetate)	0		0		0	
Carbon 2 ($BaCO_3$)	0		0		0	
" 3 "	1030*		550*		2150*	
Carbons 9, 8 (sodium acetate)	0		0			
Sum of activities of carbons 5, 3		1830		1130		3450
Pyrrole rings *C* and *D*	6600		6000		4470	
Carbon 10 ($BaCO_3$)	4600		4330		450	
Carbons 6, 4, 5 (2,4-dinitrophenylhydrazone of pyruvic acid)			760		2400	
Carbons 9, 8, 3, 2 (2,4-dinitrophenylhydrazone of α-ketobutyric acid)			800		2170	

TABLE I—*Concluded*

Position of fragment in porphyrin and compound analyzed for C^{14} activity	Citric acid-1,5-C^{14}		α-Ketoglutaric acid-5-C^{14}		α-Ketoglutaric acid-1,2-C^{14}	
	Molar activity	Sum of molar activities of fragments	Molar activity	Sum of molar activities of fragments	Molar activity	Sum of molar activities of fragments
	c.p.m.		*c.p.m.*		*c.p.m.*	
Sum of carbons 6, 4, 5, 9, 8, 3, 2, 10				5890		5020
Carbon 5 ($BaCO_3$)			590*		1710*	
Carbons 6, 4 (sodium acetate)			0		0	
Carbon 2 ($BaCO_3$)			0		0	
" 3 "			590*		1850*	
Carbons 9, 8 (sodium acetate)					0	
Sum of activities of carbons 5, 3				1180		3560
Sum of activities of carbons 3, 5, 10				5510		4010

* Since carbon atoms 6 and 4 and 9, 8, and 2 were non-radioactive, the more accurate activity figure for carbons 3 and 5 in all cases can be obtained by substituting the value found for the crystalline hydrazones of the keto acids.

qualitatively the same as that in the above experiments but quantitatively in reverse. The "active" succinate produced directly from α-ketoglutarate-1,2-C^{14} should produce a porphyrin labeled in carbon atoms 3 and 5, and the "active" succinate produced by Reaction C would give rise to a porphyrin whose carbon atoms 3, 5, and 10 would be radioactive. Therefore, again, only carbon atoms 3, 5, and 10 of the porphyrin synthesized from α-ketoglutarate-1,2-C^{14} should be radioactive, but the bulk of the activity should now reside in carbon atoms 3 and 5. It can be seen from Table I that, as predicted, all the radioactivity in the porphyrin resides in carbon atoms 3 and 5 and 10 and, further, that the activities of carbon atoms 3 and 5 are equal and much higher than that of carbon atom 10.

From theoretical and experimental considerations we have previously postulated the C^{14} distribution pattern in the "active" succinate produced by condensation of labeled acetate (methyl- or carboxyl-labeled) with oxalacetate and after one and several passages through the citric acid cycle (2). The C^{14} distribution pattern found in the porphyrin synthesized from both methyl- and carboxyl-labeled acetate was in complete agreement with the theoretical predictions. However it can be seen (from Table III, p. 329, Shemin and Wittenberg (2)) that the C^{14} distribution pattern in the

"active" succinate synthesized from methyl-labeled acetate is dependent on the number of cycles, while that synthesized from carboxyl-labeled acetate is independent of the number of cycles. If such is the case, then malonate, which can inhibit the reactions beyond the formation of succinate, should affect only the C^{14} distribution pattern in the "active" succinate synthesized from methyl-labeled acetate. This effect will then be reflected in the radioactivity and C^{14} distribution pattern of the resulting porphyrin. Therefore, malonate should lower the radioactivity in the hemin synthesized from methyl-labeled acetate and have little or no influence on the radioactivity of the hemin synthesized from carboxyl-labeled acetate. It

TABLE II

Comparison of C^{14} Activities of Hemin Synthesized from C^{14}-Methyl-Labeled Acetate and from C^{14}-Carboxyl-Labeled Acetate in Absence and Presence of Malonate

Experiment No.	Hemin synthesized from			
	C^{14}-Methyl-labeled acetate		C^{14}-Carboxyl-labeled acetate	
	Without malonate	With malonate	Without malonate	With malonate
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
1	427	249	82	67
2	499	207	87	92
3	296	125	65	54
4	283	114	56	55

In each case 25 ml. samples of washed whole duck red blood cells were used. 1.25 mg. of acetate, having an activity of 0.1 mc. per mmole, were added to each flask. The malonate concentration was 0.02 M. The concentration of glycine was 1 mg. per ml.

can be seen from Table II that, whereas the presence of malonate lowered the radioactivity in the hemin samples synthesized from methyl-labeled acetate, there was no such effect in the experiments in which carboxyl-labeled acetate was the substrate.

Further evidence of this effect of malonate on the utilization of methyl-labeled acetate for porphyrin formation was obtained by determining the C^{14} activity of the individual carbon atoms of the porphyrin molecule. In each pyrrole unit 2 moles of "active" succinate are involved (Fig. 3) (2). Therefore, if the malonate caused a complete block in the succinate-fumarate step, only carbon atoms 6 and 9 would be labeled directly, and, since Reaction C (Fig. 1) is operative, some activity will also be found in carbon atoms 4 and 8. In the ideal case of total inhibition by malonate no activity should be found in carbons 5 and 3. However, if the malonate inhibition was not complete, a small amount of activity would be found in

carbons 3 and 5, since some recycling would occur. Nevertheless, the carbon atoms most affected would be these same carbon atoms. In the absence of malonate, the radioactivities of carbons 4 and 8 and 5 and 3 are equal, as we have previously demonstrated and have confirmed in this communication (Table III). It can be seen from Table III that not only were the radioactivities of the hemin and its fragments lowered in the presence of malonate, but that of carbon 5 was much lower than that in carbon 4.

TABLE III

Distribution of C^{14} Activity in Protoporphyrin Synthesized from C^{14}-Methyl-Labeled Acetate in Absence and Presence of Malonate

Position of fragment in porphyrin and compound analyzed for C^{14} activity	Without malonate		With malonate	
	Observed activity	Molar activity	Observed activity	Molar activity
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Whole porphyrin (hemin)	3440	186,000	1000	54,000
Pyrrole rings *A* and *B* (methylethylmaleimide)	3814	88,000	1180	27,500
Carbons 6, 4, 5 of rings *A* and *B* (2,4-dinitrophenylhydrazone of pyruvic acid)	1030	45,000	310	14,000
Carbon 5	350	12,000	28	900
" 4	384	13,000	114	3,700
" 6		20,000*		9,400*

Preparations of washed duck red blood cells (680 ml.) were divided in half, each fraction was incubated with 9 mg. of acetate-2-C^{14} (0.1 mc. per mmole) and glycine (1 mg. per ml.), and one flask contained malonate (0.01 M).

* Obtained by difference.

EXPERIMENTAL

Measurement of Radioactivity—The methods were the same as those published previously, and the term "molar activity" is as previously defined (2, 3).

Biological Experiments—The duck red blood cells and the hemolyzed preparations were obtained and incubated with labeled substrates as described earlier (3, 6). Details in reference to the amounts of substrate used and their C^{14} activities and the conditions under which the incubations were done are included in Tables I to III.

C^{14}-Labeled Substrates—Acetate-1-C^{14} and acetate-2-C^{14} were purchased from Tracerlab, Inc. Citric acid-1,5-C^{14} synthesized by the method of Rothchild and Fields (7) was obtained from the Atomic Energy Commission.

α-Ketoglutaric acid-5-C^{14} was prepared in 60 to 70 per cent yield by the

method of Friedman and Kosower (8) by the condensation of diethyl succinate-1-C^{14} or diethyl succinate-1,4-C^{14} with diethyl oxalate. The labeled succinic acid was prepared as follows: (*a*) Acetic acid synthesized from $C^{14}O_2$ and methyl magnesium iodide was converted to ethyl bromoacetate by the method of Anker (9). The succinic acid was synthesized by condensation of ethyl bromoacetate with ethyl malonate. The yields of succinic acid varied from 25 to 55 per cent. (*b*) Ethylene dibromide was condensed with $KC^{14}N$ by the method of Fauconnier (10) in 80 per cent yield. The $KC^{14}N$ was prepared from $BaC^{14}O_3$ by the method of McCarter (11). The succinic acid samples were esterified with diazoethane.

The α-ketoglutaric acid, m.p. 111–113°, after recrystallization from ethylene chloride was further purified, in order to free it of any radioactive succinic acid by chromatography, on silica gel columns by methods similar to those of Marvel and Rands (12).

TABLE IV

Ceric Sulfate Decarboxylation of 2,4-Dinitrophenylhydrazone of Pyruvic Acid-2-C^{14}

Experiment No.	Molar activity of hydrazone	Molar activity of carboxyl group	Per cent contributed by α-carbon atom to evolved CO_2
	c.p.m.	*c.p.m.*	
1	223,000	17,400	7.8
2	13,400	850	6.3

α-Ketoglutaric Acid-1,2-C^{14}—Carbon dioxide generated from radioactive barium carbonate was reduced to $HC^{14}OOH$ with lithium borohydride in yields of 50 to 70 per cent by the method of Burr *et al.* (13). Oxalic acid was prepared in yields of 60 to 80 per cent from the radioactive sodium formate by fusion with sodium hydroxide at 400°. Ethyl oxalate-1,2-C^{14} was obtained by esterification of the oxalic acid with diazoethane and condensed with diethyl succinate in the presence of potassium ethoxide (8).

Degradation of Labeled Hemin

The hemin, isolated by methods previously described, was degraded by the chemical methods (2, 5) with one modification. The hydrazones of the keto acids were degraded with ceric sulfate (14) rather than with permanganate. Ceric sulfate was used in order to avoid the 7-fold dilution of the carboxyl group due to oxidation of the phenyl group to carbon dioxide. It was found that in the degradation with ceric sulfate the carboxyl group liberated as carbon dioxide is contaminated to the extent of 5 to 10 per cent by the α-carbon atom as determined on a sample of pyruvic acid-2-C^{14} hydrazone (Table IV). Therefore, we have discounted any

activity of less than 10 c.p.m. in carbon atoms 2 as having any biological significance. It is comforting, however, to find that the sum of the activities of the radioactive carbon atoms is equal to that of the parent compound.

SUMMARY

The utilization of citric acid-1,5-C^{14}, α-ketoglutaric acid-5-C^{14}, and α-ketoglutaric acid-1,2-C^{14} for porphyrin formation in hemolyzed preparations of duck red blood cells was studied. In each instance, those carbon atoms of the porphyrin which were predicted on the basis of the formation of "active" succinate in the citric acid cycle to contain C^{14} were radioactive. Further, the C^{14} distribution patterns found in the porphyrin were in complete agreement with our previous formulations regarding porphyrin formation from "active" succinate and glycine.

It was found that malonate influenced the utilization of C^{14} methyl-labeled acetate for porphyrin formation, while the presence of malonate did not affect the utilization of C^{14} carboxyl-labeled acetate. This is in agreement with the postulation that the C^{14} distribution pattern found in the "active" succinate synthesized from methyl-labeled acetate via the citric acid cycle is dependent on the number of turns occurring in the cycle, while that synthesized from C^{14} carboxyl-labeled acetate is independent of the number of turns.

BIBLIOGRAPHY

1. Wriston, J. C., Jr., Lack, L., and Shemin, D., *Federation Proc.*, **12,** 294 (1953).
2. Shemin, D., and Wittenberg, J., *J. Biol. Chem.*, **192,** 315 (1951).
3. Shemin, D., and Kumin, S., *J. Biol. Chem.*, **198,** 827 (1952).
4. London, I. M., and Yamasaki, M., *Federation Proc.*, **11,** 250 (1952).
5. Wittenberg, J., and Shemin, D., *J. Biol. Chem.*, **185,** 103 (1950).
6. Shemin, D., London, I. M., and Rittenberg, D., *J. Biol. Chem.*, **183,** 757 (1950).
7. Rothchild, S., and Fields, M., *J. Am. Chem. Soc.*, **74,** 2401 (1952).
8. Friedman, L., and Kosower, E., *Org. Syntheses*, **26,** 42 (1946).
9. Anker, H. S., *J. Biol. Chem.*, **176,** 1333 (1948).
10. Fauconnier, A., *Bull. Soc. chim.*, **50,** 214 (1888).
11. McCarter, J. A., *J. Am. Chem. Soc.*, **73,** 483 (1951).
12. Marvel, C. S., and Rands, R. D., Jr., *J. Am. Chem. Soc.*, **72,** 2642 (1950).
13. Burr, J. G., Jr., Brown, W. G., and Heller, H. E., *J. Am. Chem. Soc.*, **72,** 2560 (1950).
14. Corzo, R. H., and Tatum, E. L., *Federation Proc.*, **12,** 470 (1953).

THE SUCCINATE-GLYCINE CYCLE*

I. THE MECHANISM OF PYRROLE SYNTHESIS

By DAVID SHEMIN, CHARLOTTE S. RUSSELL, AND TESSA ABRAMSKY

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York)

(Received for publication, January 10, 1955)

We have recently published a series of reactions which appear to be concerned with the metabolism of glycine (1). In this group of reactions, called the succinate-glycine cycle (Fig. 1), "active" succinate condenses on the α-carbon atom of glycine to yield α-amino-β-ketoadipic acid. This β-keto acid on decarboxylation yields δ-aminolevulinic acid, in which the δ-carbon atom is originally the α-carbon atom of glycine (1, 2). The aminoketone, which serves as the source for all the carbon and nitrogen atoms of the porphyrin molecule,[1] is also metabolized to yield its δ-carbon atom for the ureido groups of purines (1) and for the β-carbon atom of serine for methyl groups and is converted to formic acid[2] (3). This metabolic pattern for the δ-carbon atom of δ-aminolevulinic acid is the same as that known for the α-carbon atom of glycine. These findings provide a pathway of glycine metabolism by which these dissimilar compounds are metabolically related. Further, the succinate-glycine cycle provides a pathway by which glycine is converted to carbon dioxide. The original carboxyl group of glycine is converted to carbon dioxide on decarboxylation of α-amino-β-ketoadipic acid, and the α-carbon atom of the glycine is converted to carbon dioxide subsequent to the conversion of the δ-aminolevulinic acid to α-ketoglutaric acid[2] (3). The α-carboxyl group of the latter compound arises from the α-carbon atom of glycine, and on oxidative de-

* This work was supported by grants from the National Institutes of Health, United States Public Health Service (RG-1128(C6)), from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and from the Rockefeller Foundation.

[1] The succinate moiety of δ-aminolevulinic acid is utilized for the same carbon atoms of the protoporphyrin molecule (Schiffman, E., and Shemin, D., unpublished results) as is succinate (4).

[2] It was found that the methyl group of methionine (Foster, G. L., and Shemin, D., unpublished results) and the β-carbon atom of serine (Gatt, S., and Shemin, D., unpublished results) were radioactive after injection of δ-aminolevulinic acid-5-C^{14} into a duck. The administration by injection of δ-aminolevulinic acid-5-C^{14} into a rat resulted in the urinary excretion of highly radioactive formic acid (Gatt, S., and Shemin, D., unpublished results). Also α-ketoglutaric acid contained C^{14} activity after incubation of a duck red blood cell hemolysate with δ-aminolevulinic acid-5-C^{14}.

XUM

carboxylation of the ketoglutarate a 2nd mole of carbon dioxide is formed. The succinate-glycine cycle is similar to the citric acid cycle in that they are both concerned with the metabolism of a 2-carbon compound. Oxalacetate is the substrate catalyst in the citric acid cycle, whereas succinate plays this rôle in the reactions in which glycine is concerned.

This communication is concerned with only part of the succinate-glycine cycle; namely, the conversion of δ-aminolevulinic acid to porphyrin.

We have previously demonstrated the source of each of the carbon atoms of protoporphyrin, 26 being derived from "active" succinate (4, 5) and 8 from the α-carbon atom of glycine (6–8). These two compounds are found in particular positions in the porphyrin; the α-carbon atom of glycine is the source of the methene bridge carbon atom (7, 8) and the 2 position in each of the pyrrole rings (7) (Fig. 2 of the preceding paper (9)). It is also noteworthy that the carboxyl group of glycine is not utilized for porphyrin synthesis (6, 10). This finding was an important clue in the elucidation of the mechanism of condensation of the succinate and glycine.

Until recently the mechanism by which succinate and glycine condense to form the pyrrole unit was not known. Any explanation of this condensation should not only be concerned with pyrrole formation, but should also afford a reasonable biochemical mechanism by which the carboxyl group of glycine is separated from its α-carbon atom. Further, the mechanism of pyrrole formation should account for the positional distribution of the α-carbon atom of glycine in the porphyrin molecule. These considerations must be included in any approach because the α-carbon atom of glycine is *equally* distributed in the 2 position of the pyrrole rings and in the methene bridges, and because no "C_1" compound has been found to substitute for glycine (3). It is therefore evident that the whole molecule of glycine is involved in its initial condensation with succinate. If the "active" succinate condensed on the α-carbon atom of glycine to form a new intermediate, α-amino-β-ketoadipic acid, all the experimental findings and conclusions would be satisfied (Fig. 1). This compound, being a β-keto acid, can then readily be decarboxylated, and thus a mechanism is provided for the utilization of the whole molecule of glycine in the initial step and a mechanism for subsequent separation of the carboxyl group of glycine from its α-carbon atom. Further, condensation of 2 moles of the resulting compound, δ-aminolevulinic acid, by a Knorr type of mechanism would yield a pyrrole in which the α-carbon atom of glycine would be in the 2 position and in the α substituent (aminomethyl) of the pyrrole, and would thus constitute the source of the methene bridge carbon atoms (Fig. 2). This formulation is supported by our recent finding that the molar radioactivity of porphobilinogen enzymatically synthesized from δ-aminolevulinic acid-5-C^{14} is twice that of the latter compound (11).

In order to test the above hypothesis, δ-aminolevulinic acid was synthesized and its rôle in the biosynthesis of protoporphyrin was investigated. In the initial experiments, non-radioactive δ-aminolevulinic acid was added to a duck red blood cell hemolysate along with either C^{14}-labeled succinate or with glycine-2-C^{14}. If δ-aminolevulinic acid is formed as an inter-

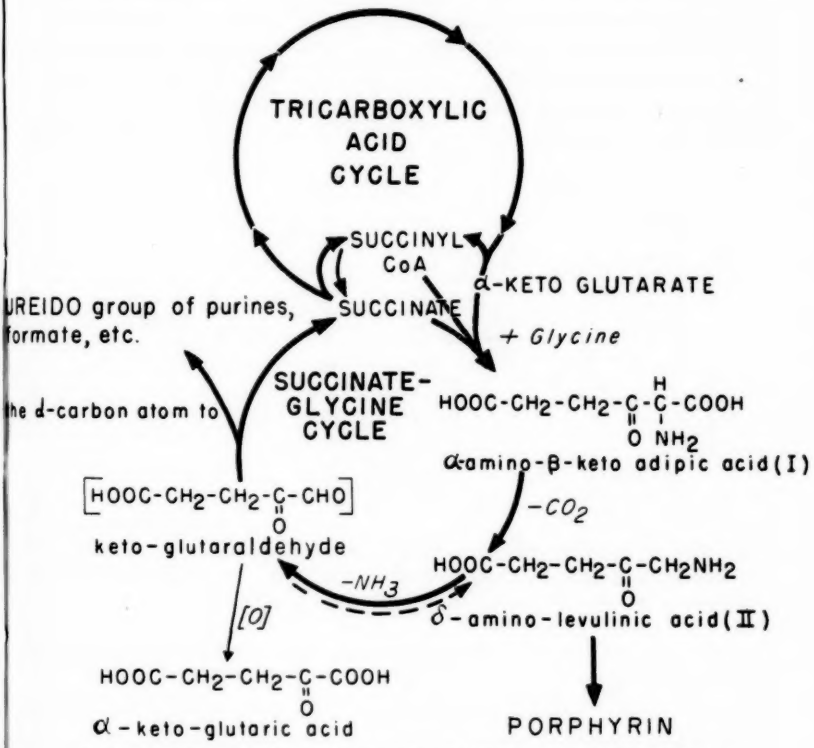


FIG. 1. Succinate-glycine cycle; a pathway for the metabolism of glycine

mediate[3] in the biosynthesis of porphyrins from succinate and glycine, the addition of unlabeled δ-aminolevulinic acid should theoretically be reflected in a lowered C^{14} activity of the newly formed heme, since a dilution would occur. It can be seen from Table I, in which a few of the experiments are given, that the C^{14} activities of the isolated hemin samples synthesized from either C^{14}-labeled succinate or from C^{14}-labeled glycine in the presence of

[3] On incubation of hemolysates with glycine-2-C^{14} in the presence of unlabeled δ-aminolevulinic acid, the formaldehyde formed on treating the protein-free supernatant fluid with periodic acid was highly radioactive (see "Experimental").

XUM

non-radioactive δ-aminolevulinic acid were much lower than those of the experiments in which the diluent was not added. This effect was not due to an inhibition, since in Experiment 2, Table I, in which the δ-aminolevulinic acid was labeled with N^{15}, there was a simultaneous excellent incorporation of N^{15} into the porphyrin. The incorporation into heme of N^{15} from the δ-aminolevulinic acid was much greater than that found in the experiment in which 6 to 7 times as much N^{15}-labeled glycine was the substrate.

This utilization of δ-aminolevulinic acid for porphyrin formation was further demonstrated by incubation of the duck red blood cell hemolysates with δ-aminolevulinic acid-5-C^{14}. As can be seen from Table II, not only did the hemin samples contain C^{14}, but their radioactivities were about

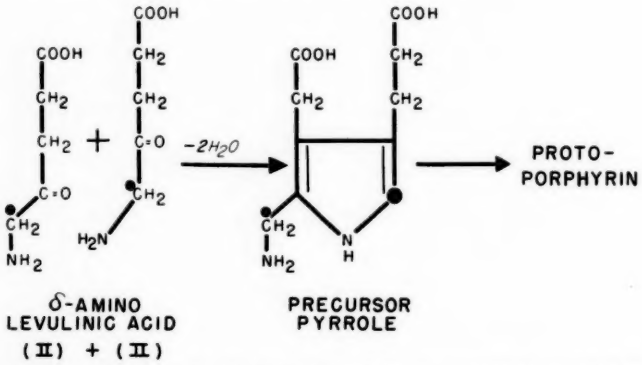


FIG. 2. The mechanism for the formation of the monopyrrole, porphobilinogen, by condensation of 2 moles of δ-aminolevulinic acid. The carbon atoms bearing the closed circles (●) were originally the α-carbon atom of glycine.

65 times greater than those of samples made from glycine-2-C^{14} having the same C^{14} activity.

Further proof (2) that δ-aminolevulinic acid was synthesized by the reaction outlined in Fig. 1 and that it was an intermediate in porphyrin synthesis was obtained by degradation of hemin samples synthesized from δ-aminolevulinic acid-5-C^{14}. The δ-carbon atom of the latter compound should, according to the hypothesis, metabolically arise from the α-carbon atom of glycine. Thus the C^{14} distribution pattern among the carbon atoms of protoporphyrin synthesized from δ-aminolevulinic acid-5-C^{14} should be exactly the same as that previously found for glycine-2-C^{14}; 50 per cent of the activity should reside in the four pyrrole rings and 50 per cent should reside in the 4 methene bridge carbon atoms (Fig. 3). It can be seen from Table III that the same C^{14} distribution pattern in the porphyrin was realized for δ-aminolevulinic acid-5-C^{14} and glycine-2-C^{14}.

The utilization of δ-aminolevulinic acid for porphyrin formation provides

the evidence for the condensation of "active" succinate and glycine to form α-amino-β-ketoadipic acid. The latter compound is therefore an obligatory intermediate in porphyrin formation,[4] and its decarboxylation to δ-aminolevulinic acid provides the mechanism by which the carboxyl group

TABLE I

Comparison of C^{14} Activities of Hemin Samples Synthesized from Glycine-2-C^{14} (0.05 mc. per mmole) or Succinic Acid-2-C^{14} (0.05 mc. per mmole) in Presence and Absence of Non-Radioactive δ-Aminolevulinic Acid

Experiment No.	Substrates			Isotope concentration in hemin	
	C^{14}-labeled	N^{15}-labeled	Unlabeled	C^{14}	N^{15}
				c.p.m.	*atom per cent excess*
1	Glycine-2-C^{14} (0.05 mmole)			125	
	Glycine-2-C^{14} (0.05 mmole)		δ-Aminolevulinic acid (0.05 mmole)	15	
2	Glycine-2-C^{14} (0.05 mmole)			230	
	Glycine-2-C^{14} (0.05 mmole)	δ-Aminolevulinic acid (0.05 mmole)*		48	0.21
		Glycine (0.33 mmole)*			0.06
3	Succinate-2-C^{14} (0.1 mmole)			660	
	Succinate-2-C^{14} (0.1 mmole)		δ-Aminolevulinic acid (0.1 mmole)	180	

* The isotopic concentration of these samples was 34 atom per cent excess N^{15}. In each of the experiments the volume of the hemolyzed preparation was 30 ml. Unlabeled succinate (0.1 mmole) was added to the flasks in which labeled glycine was the substrate, and unlabeled glycine (0.33 mmole) was added to the flasks in which labeled succinate was the substrate. Each flask contained 1 mg. of iron (ferric).

of glycine is detached from its α-carbon atom. Subsequent to the initial publication (1) of the conversion of δ-aminolevulinic acid to protoporphyrin, Neuberger and Scott (12) and later Dresel and Falk (13) published experiments which were in agreement with this observation.

It is also of interest to note the degree of organization of the duck red

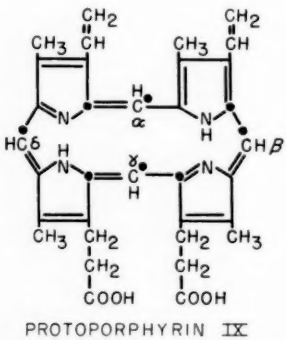
[4] The intraperitoneal injection into a rat of the diethyl ester of α-amino-β-ketoadipic acid gives rise to porphobilinogen, as does the injection of δ-aminolevulinic acid (Weliky, I., and Shemin, D., unpublished observations).

blood cell preparation necessary for the condensation of the "active" succinate with glycine to form eventually δ-aminolevulinic acid. It has been demonstrated that not only can whole red cells (14) effect the synthesis of porphyrin from these two substrates, but also that the synthesis occurs in a gently hemolyzed preparation (4, 15, 16). However, more drastic disruption of the preparation, namely homogenization in a Waring blendor,

TABLE II

Comparison of C^{14} Activities of Hemin Samples Synthesized from Glycine-2-C^{14} (0.05 mc. per mmole) and δ-Aminolevulinic Acid (0.05 mc. per mmole)

C^{14} substrate	Other additions	C^{14} activity of hemin
		c.p.m.
Glycine-2-C^{14} (0.05 mmole)	Succinate (0.1 mmole)	333
δ-Aminolevulinic acid (0.05 mmole)		23,000
" " (0.05 ")	Glycine (0.33 mmole) + succinate (0.1 mmole)	21,000



$NH_2-\overset{\bullet}{C}H_2-COOH$

Glycine-2-C^{14}

$NH_2-\overset{\bullet}{C}H_2-CO-CH_2-CH_2-COOH$

δ-Aminolevulinic Acid-5-C^{14}

FIG. 3. The carbon atoms of protoporphyrin which arise from the α-carbon atom of glycine and from the δ-carbon atom of δ-aminolevulinic acid.

causes the system to lose most if not all of its synthetic capacity for heme synthesis when succinate or ketoglutarate and glycine are the substrates (Table IV). On the other hand, we have found that the homogenate can synthesize heme when δ-aminolevulinic acid is the substrate (2). Further, even a particle-free extract obtained by high speed centrifugation, and a lyophilized powder of the extract, can synthesize the porphyrin from δ-aminolevulinic acid (Table IV). It appears, therefore, that a solution of enzymes can synthesize the porphyrin from δ-aminolevulinic acid and that on homogenization the functional activity of only those enzymes concerned

in the condensation of "active" succinate with glycine is lost. Since the conversion of δ-aminolevulinic acid (2) and porphobilinogen (11, 17, 18) to porphyrins is now known to occur in a soluble enzyme system, the possibility has been opened up of isolating the enzymes concerned with the individual steps and thus studying the reactions in greater detail (11). Indeed, it has been found subsequently that a highly purified protein fraction obtained from ox liver (19) and from duck erythrocytes (11) can catalyze the conversion of δ-aminolevulinic acid to porphobilinogen.

TABLE III

Distribution of C^{14} Activity in Protoporphyrin Synthesized from δ-Aminolevulinic Acid-5-C^{14} and from Glycine-2-C^{14}

Fragments of porphyrin	C^{14} activity in fragments of porphyrin synthesized from			
	δ-Aminolevulinic acid-5-C^{14}			Glycine-2-C^{14} (7)
	Found	Molar activity	Molar activity	Molar activity
	c.p.m.	*c.p.m.*	*per cent*	*per cent*
Whole porphyrin	1174	55,400	100	100
Pyrrole ring *A* + *B* (methylethylmaleimide)	586	13,600	24.6	24.6
Pyrrole ring *C* + *D* (hematinic acid)	454	13,900	25.1	25.3
Pyrrole ring *A* + *B* + *C* + *D*		27,500	49.7	49.9
Methene bridge carbon atoms		27,900*	50.3*	50.1*

* Obtained by difference. In the experiment in which δ-aminolevulinic acid-5-C^{14} was the substrate, the CO_2 was collected on chromic acid oxidation of the mesoporphyrin. Although most of the evolved CO_2 arises from the methene bridge carbon atoms, side reactions contribute some CO_2. However, the evolved CO_2 was radioactive (375 c.p.m.) and its molar activity therefore was 20,500 c.p.m.

EXPERIMENTAL

Synthesis of δ-Aminolevulinic Acid Hydrochloride

The details of the three methods of synthesis, previously announced (1), are given below. Prior to the development of our methods, Wynn and Corwin synthesized the compound by still another procedure (20).

Preparation A. Synthesis from β-Ketoadipic Acid—Ethyl β-keto-α-carbethoxyadipate was prepared according to the method of Riegel and Lilienfeld (21) by the condensation of the ethylmagnesiomalonate synthesized (22) from 8 gm. of diethyl malonate with 9.3 gm. of β-carbethoxypropionyl chloride synthesized from succinic anhydride. The triester, purified by distillation, was hydrolyzed to β-ketoadipic acid with concentrated hydro-

chloric acid for 18 hours at room temperature, according to the method of Eisner, Elvidge, and Linstead (23). The crude crystalline β-ketoadipic acid was suspended in 50 ml. of glacial acetic acid, and an equimolar amount of ethyl nitrite (24) was passed at 15–20° into the suspension over a period of about 20 minutes. After standing at room temperature for 1 hour, during which time all the crystalline material dissolved, the solution was taken to dryness *in vacuo* at 30–35° and the oily residue added dropwise to an ice-

TABLE IV

*Comparison of C^{14} Activities of Hemin Synthesized from δ-Aminolevulinic Acid-5-C^{14} (0.05 mc. per mmole), from Glycine-2-C^{14} (0.05 mc. per mmole), and from Succinic Acid-2-C^{14} (0.05 mc. per mmole) in Different Duck Red Blood Cell Preparations**

Experiment No.	C^{14} substrate	Red cell preparation	C^{14} activity in hemin sample
1	Glycine (0.05 mmole)†	Hemolyzed	395
	" (0.05 ")†	Homogenized	2
	Succinate (0.05 mmole)‡	Hemolyzed	565
	" (0.05 ")‡	Homogenized	1
	δ-Aminolevulinic acid (0.006 mmole)	Hemolyzed	3900
	" " (0.006 ")	Homogenized	4500
2	" " (0.009 ")	"	2200
	" " (0.009 ")	Supernatant ($12 \times 10^3 \times g$)	1600
	" " (0.009 ")	" ($47 \times 10^3 \times$ ")	1600
	" " (0.009 ")	" ($100 \times 10^3 \times$ ")	1500
3	" " (0.009 ")	" ($12 \times 10^3 \times$ ")	2000
	" " (0.009 ")	Lyophilized preparation	1500

* Each preparation represented 25 ml. of duck blood prepared as previously described (4, 14) and contained 1 mg. of iron (ferric).

† Plus 0.1 mmole of non-radioactive α-ketoglutaric acid.

‡ Plus 0.33 mmole of non-radioactive glycine.

cold solution of 25 gm. of $SnCl_2 \cdot 2H_2O$ in 25 ml. of concentrated hydrochloric acid. The resulting solution, after standing for 48 hours in the refrigerator, was diluted to 500 ml. with water and treated with hydrogen sulfide. The tin sulfide was filtered off and washed with water. The filtrate and washings were taken to dryness *in vacuo*, and the residue was dried in a desiccator over NaOH. The crystalline residue was dissolved in a minimum of ethanol and recrystallized by the addition of ethyl acetate. The white crystalline material was further purified from alcohol-ethyl acetate; yield 2.0 to 2.2 gm.

The δ-aminolevulinic acid hydrochloride, m.p. 149–151°, gave a positive ninhydrin test, reduced Benedict's solution in the cold, and gave one spot

on an ascending paper chromatogram with an R_F of about 0.42 to 0.44 in a phenol-water mixture.

$C_5H_9O_3N \cdot HCl$.	Calculated.	C 35.8, H 6.0, N 8.4, Cl 21.2
	Found.	" 35.6, " 6.7, " 8.3, " 21.0

For the synthesis of δ-aminolevulinic acid-5-C^{14}, diethyl malonate-2-C^{14} was used, and for the synthesis of N^{15}-labeled δ-aminolevulinic acid, ethyl nitrite prepared from $KN^{15}O_2$ was used to form the isonitroso derivative. The $KN^{15}O_2$ was prepared from $KN^{15}O_3$.

Preparation B. By Gabriel Synthesis—To an ethereal solution of 1 gm. of β-carbomethoxypropionyl chloride was added an ethereal solution of diazomethane obtained from 2 gm. of *N*-nitroso-*N*-methylurea, and the mixture was stirred for 2 hours at room temperature. After this time either dry HCl gas was bubbled through the solution or an ethereal solution of HCl was added dropwise until the evolution of N_2 ceased. The solvent was removed by passing a dry stream of nitrogen through the solution, and the residue was dried *in vacuo* over NaOH. The oily residue was treated with 4 ml. of dimethylformamide and 1.24 gm. of potassium phthalimide (labeled with 34 atom per cent excess N^{15}). The resulting orange suspension was stirred vigorously for 1 hour at room temperature and then dissolved in 25 ml. of chloroform. The chloroform solution was washed with 50 ml. of water and the washings shaken with 2 ml. portions of chloroform. The combined chloroform solutions were then washed twice with 50 ml. portions of water, once with 25 ml. of 0.2 N sodium hydroxide, and finally four times with water. The chloroform solution was dried by filtration and taken to dryness *in vacuo*. The crude methyl δ-phthalimidolevulinate was purified by crystallization from methanol, m.p. 96–97°. The phthalimido derivative was then hydrolyzed by boiling for 7 to 10 hours with 6 N hydrochloric acid. After removal of the phthalic acid, the crude δ-aminolevulinic acid hydrochloride was purified as in Preparation A. Neuberger and Scott recently independently synthesized this aminoketone by a similar procedure (25).

The material of this synthesis had the same characteristic properties as those described in the above preparation, and its melting point was not depressed when mixed with a sample obtained in Preparation A.

Preparation C. From Urocanic Acid[5]—2 gm. of urocanic acid in aqueous ethanol were reduced to imidazolepropionic acid with palladium and hydrogen. The acid was converted to its methyl ester with dry methanolic hydrochloric acid and then exhaustively benzoylated by the method of

[5] We wish to thank Dr. H. Tabor of the National Institutes of Health, Bethesda, for his generous gift of urocanic acid.

Ashley and Harington (26) as modified by Tesar and Rittenberg (27). The dibenzoyl derivative obtained from the benzene layer was purified by recrystallization from methanol-water. 800 mg. of material, m.p. 136–142°, were obtained.

500 mg. were hydrolyzed by boiling with 20 ml. of dry 10 per cent methanolic HCl for 1.5 hours. The residue obtained after distillation *in vacuo* was then refluxed with 20 ml. of 6 N hydrochloric acid for another 1.5 hours. The crystals which were formed on cooling were removed by filtration, and the filtrate was taken to dryness and purified by crystallization from alcohol-ethyl acetate solution. 60 mg. of δ-aminolevulinic acid hydrochloride were obtained, m.p. 149–151°, having the same R_F as samples prepared by the foregoing methods and the same chemical characteristics.

No depression in melting point was observed when the compound was mixed with a sample from Preparation A.

Periodate Treatment—In order to secure further support of the structure assigned to the compounds obtained from the three independent syntheses, samples of δ-aminolevulinic acid were treated with periodate. Above pH 7, 1 mole of periodate was taken up, and from the reaction products a 95 per cent yield of formaldehyde, isolated as the dimedon derivative, m.p. 192°, and a 90 per cent yield of succinic acid, m.p. 190–191°, were obtained. Mixed melting points with authentic samples gave no depression in the melting point.

Hemolyzed Duck Red Blood Cells—These preparations were made as described previously, and the same additions were made (4).

Preparation of Extract—The cold hemolyzed preparations were homogenized in a cold Waring blendor for 10 minutes, and the mixture was then centrifuged for 30 minutes to 4 hours in a Spinco preparative centrifuge at gravitational forces indicated in Table IV. This extract was used, without further additions, in the experiments.

Incubation Experiments—The hemolyzed preparations and the extracts were shaken at room temperature, usually for 16 hours. The additions and radioactivities of the substrates are specified in Tables I to IV.

Degradation of Labeled Protoporphyrin—The radioactive hemin isolated was degraded to methylethylmaleimide and hematinic acid by procedures previously described (5, 7).

Measurement of Radioactivity—The methods employed were published previously, and the use of the term "molar activity" is as defined earlier (4, 5).

DISCUSSION

The results obtained in this study of the conversion of δ-aminolevulinic acid into protoporphyrin are in complete agreement with the conclusion

that "active" succinate and glycine condense to form α-amino-β-ketoadipic acid, and the latter keto acid then decarboxylates to form δ-aminolevulinic acid. The distribution of the δ-carbon atom of this aminoketone among the carbon atoms of the porphyrin supports the suggested mechanism for the formation of the monopyrrole. The mechanism pictured is the condensation of 2 moles of δ-aminolevulinic acid by a Knorr type of condensation to form the precursor pyrrole (Fig. 2); this has subsequently been supported by the finding that the molar radioactivity of porphobilinogen enzymatically synthesized from δ-aminolevulinic acid-5-C^{14} is twice that of the latter compound (11). It is of interest that the structure of the precursor pyrrole arrived at from these theoretical considerations is the same as that determined for porphobilinogen (28, 29).

All the previous data were consistent with the suggestion that a precursor pyrrole is first formed and subsequently utilized for synthesis of the tetrapyrrole compound. The previous considerations and the findings in this communication, coupled with those dealing with the structure of porphobilinogen, and the experiments demonstrating the formation of porphobilinogen from δ-aminolevulinic acid and its conversion to porphyrins in biological systems (11, 12, 17–19) adequately demonstrate that the monopyrrole porphobilinogen is indeed an intermediate in porphyrin synthesis.

Although no evidence has yet been obtained concerning the biological mechanism of conversion of the monopyrrole to the tetrapyrrole structure, several suggestions have been advanced (17, 30). We should like to suggest still another possibility which may explain the distribution of the α-carbon atom of glycine or the δ-carbon atom of δ-aminolevulinic acid in the porphyrin molecule of the I and III series. It is based on the synthetic mechanism of dipyrrole and tetrapyrrole formation demonstrated by Corwin *et al.* (31, 32) and is similar to that postulated by Bogorad and Granick (17).

Condensation of 3 moles of the precursor pyrrole (porphobilinogen), or of a closely related derivative, by a mechanism of Corwin *et al.* (31, 32) would lead to a tripyrrylmethane compound, as schematically represented in Fig. 4. The tripyrrylmethane then breaks down into a dipyrrylmethane and a monopyrrole. The structure of the dipyrrylmethane is dependent on the place of splitting. An *A* split would give rise to dipyrrylmethane *A*, and a *B* split would give rise to dipyrrylmethane *B*. Condensation of 2 moles of dipyrrylmethane *A* would give rise to a porphyrin of the I series, while condensation of a mole of *A* and a mole of *B* would give rise to a porphyrin of the III series. In the formation of the porphyrin of the III series it can be seen from Fig. 4 that it is necessary to lose a 1-carbon compound, since there are three aminomethyl side chains and only two are required to condense the two dipyrroles to the porphyrin structure. If the

mechanism similar to that outlined in Fig. 4 is concerned with porphyrin synthesis, it would appear that this 1-carbon compound given off could well be formaldehyde. Consistent with this idea is our finding[6] that on the conversion of porphobilinogen to porphyrins, either by heating under acid conditions (27) or by enzymatic conversion in cell-free extracts (2), formaldehyde was formed. This was established by heating or incubating porphobilinogen labeled with C^{14} in the aminomethyl group (11) and isolating radioactive formaldehyde as the dimedon derivative. It is difficult at present to speculate as to a uniform mechanism concerning the formation of the tetrapyrrole structures, but we should like to suggest that in the intermediate stages in the formation of protoporphyrin at least some of the bridge atoms are methylene groups and that it is in such intermediates that

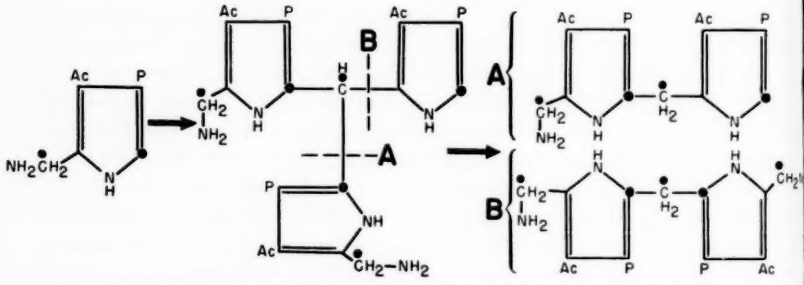


FIG. 4. A mechanism of porphyrin formation from the monopyrrole. Ac = acetic acid side chain; P = propionic acid side chain; ● = α-carbon atom of glycine and δ-carbon atom of δ-aminolevulinic acid.

decarboxylation occurs. This is consistent with our finding that coproporphyrin III is not converted to protoporphyrin in a duck red blood hemolysate or extract.[7]

SUMMARY

The biological mechanism concerned with the utilization of "active" succinate and glycine for porphyrin formation was investigated. It was concluded that the succinate condenses on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid. This keto acid undergoes decarboxylation to yield δ-aminolevulinic acid. Condensation of 2 moles of the aminoketone forms the precursor monopyrrole. This series of reactions provides

[6] Shemin, D., and Schmid, R., unpublished results.

[7] We have synthesized C^{14}-labeled coproporphyrin III, and in all our experiments we have been unable to demonstrate its conversion to protoporphyrin (Russell, C. S., and Shemin, D., unpublished results).

the mechanism by which the carboxyl group of glycine is detached from its α-carbon atom and provides the mechanism for pyrrole formation. These conclusions were reached from studies in which the utilization of δ-aminolevulinic acid for porphyrin formation was investigated. It was found that unlabeled δ-aminolevulinic acid markedly lowered the C^{14} activity of protoporphyrin synthesized by duck red blood cell hemolysates from either C^{14}-labeled succinate or C^{14}-labeled glycine. Also δ-aminolevulinic acid-5-C^{14} gave rise to labeled protoporphyrin whose C^{14} distribution pattern was the same as that found in protoporphyrin synthesized from glycine-2-C^{14}. This is in agreement with the postulated reactions, since the δ-carbon atom of δ-aminolevulinic acid should arise from the α-carbon atom of glycine.

It was also found that, whereas a homogenate of duck red blood cells is incapable of forming porphyrin from succinate and glycine, it can synthesize protoporphyrin from δ-aminolevulinic acid. Further, a particle-free extract of duck red blood cells can synthesize porphyrin from δ-aminolevulinic acid, even after lyophilization. It would appear that those enzymes which can convert δ-aminolevulinic acid to protoporphyrin are soluble and that homogenization destroys the functional activity of only those enzymes concerned with the condensation of "active" succinate and glycine.

The formation of porphyrin is discussed, and consistent with the theory is the finding that on conversion of porphobilinogen to porphyrin formaldehyde is formed from the aminomethyl group of the porphobilinogen.

BIBLIOGRAPHY

1. Shemin, D., and Russell, C. S., *J. Am. Chem. Soc.*, **75,** 4873 (1953).
2. Shemin, D., Abramsky, T., and Russell, C. S., *J. Am. Chem. Soc.*, **76,** 1204 (1954).
3. Shemin, D., in McElroy, W. D., and Glass, B., Amino acid metabolism, Baltimore, **4,** 727 (1954).
4. Shemin, D., and Kumin, S., *J. Biol. Chem.*, **198,** 827 (1952).
5. Shemin, D., and Wittenberg, J., *J. Biol. Chem.*, **192,** 315 (1951).
6. Radin, N. S., Rittenberg, D., and Shemin, D., *J. Biol. Chem.*, **184,** 745 (1950).
7. Wittenberg, J., and Shemin, D., *J. Biol. Chem.*, **185,** 103 (1950).
8. Muir, H. M., and Neuberger, A., *Biochem. J.*, **47,** 97 (1950).
9. Wriston, J. C., Jr., Lack, L., and Shemin, D., *J. Biol. Chem.*, **215,** 603 (1955).
10. Grinstein, M., Kamen, M. D., and Moore, C. V., *J. Biol. Chem.*, **174,** 767 (1948).
11. Schmid, R., and Shemin, D., *J. Am. Chem. Soc.*, **77,** 506 (1955).
12. Neuberger, A., and Scott, J. J., *Nature*, **172,** 1093 (1953).
13. Dresel, E. I. B., and Falk, J. E., *Nature*, **172,** 1185 (1953).
14. Shemin, D., London, I. M., and Rittenberg, D., *J. Biol. Chem.*, **183,** 757 (1950).
15. London, I. M., and Yamasaki, M., *Federation Proc.*, **11,** 250 (1952).
16. Dresel, E. I. B., and Falk, J. E., *Biochem. J.*, **56,** 156 (1954).
17. Bogorad, L., and Granick, S., *Proc. Nat. Acad. Sc.*, **39,** 1176 (1953).
18. Falk, J. E., Dresel, E. I. B., and Rimington, C., *Nature*, **172,** 292 (1953).
19. Gibson, K. D., Neuberger, A., and Scott, J. J., *Biochem. J.*, **58,** p. xli (1954).
20. Wynn, R. W., and Corwin, A. H., *J. Org. Chem.*, **15,** 203 (1950).

21. Riegel, B., and Lilienfeld, W. M., *J. Am. Chem. Soc.*, **67,** 1273 (1945).
22. Lund, H., and Voigt, A., *Org. Syntheses*, coll. **2,** 594 (1943).
23. Eisner, U., Elvidge, J. A., and Linstead, R. P., *J. Chem. Soc.*, 2223 (1950).
24. Semon, W. L., and Damerell, V. R., *Org. Syntheses*, coll. **2,** 204 (1943).
25. Neuberger, A., and Scott, J. J., *J. Chem. Soc.*, 1820 (1954).
26. Ashley, J. N., and Harington, C. R., *J. Chem. Soc.*, 2586 (1930).
27. Tesar, C., and Rittenberg, D., *J. Biol. Chem.*, **170,** 35 (1947).
28. Westall, R. G., *Nature*, **170,** 614 (1952).
29. Cookson, G. H., and Rimington, C., *Nature*, **171,** 875 (1953).
30. Cookson, G. H., and Rimington, C., *Biochem. J.*, **57,** 476 (1954).
31. Corwin, A. H., and Andrews, J. S., *J. Am. Chem. Soc.*, **59,** 1973 (1937).
32. Andrews, J. S., Corwin, A. H., and Sharp, A. G., *J. Am. Chem. Soc.*, **72,** 491 (1950).

16-SUBSTITUTED STEROIDS

XII. ESTRADIOL-3,16α

By MAX N. HUFFMAN and MARY HARRIET LOTT

(From the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma)

(Received for publication, January 20, 1955)

In the reduction of 1,3,5(10)-estratrien-3-ol-16-one (1) by sodium borohydride, or by hydrogen and Adams' catalyst, one epimeric estradiol-3,16 is formed preferentially (2). We have tentatively designated this epimer as estradiol-3,16β. For the reduction of 16-ketoestradiol by hydrogen and Adams' catalyst, as well as by sodium borohydride, yields 16-epiestriol, the configuration of which at C_{16} is known to be β, according to the modern terminology for ring D (1). The other 16-epimer, estradiol-3,16α, may be readily obtained by epimerizing the C_{16}-β-alcohol in the *p*-toluenesulfonate-acetate exchange reaction. This transformation is described in detail in this publication.

Estradiol-3,16α has been characterized as the diacetate, the dibenzoate, the 3-monobenzoate, and the 3-methyl ether-16-acetate. Table I shows a comparison of the properties of estradiol-3,16β and estradiol-3,16α. It is to be noted that estradiol-3,16α is definitely more dextrorotatory than is the β epimer. The two epimers give a marked mixed melting point depression. A distinguishing characteristic of estradiol-3,16α is that this diol, unless rigorously pure, furnishes an olive-green coloration at its melting point in the capillary tube. We have never prepared a sample which did not show at least a faint bluish green coloration if the melting point tube was observed against a white background. The longer a sample of estradiol-3,16α stands on the laboratory shelf, the more coloration it shows at its melting point, the shift in color being from bluish green to olive-green.

Estradiol-3,16α is of biological interest; it is in fact the 17-desoxy analogue of natural estriol. Whereas estradiol-3,16β has negligible estrogenic activity (2), estradiol-3,16α has considerable, being approximately one-fourth as active as estrone.[1] It is interesting to note that the ring D displacement of the carbinol of the β configuration from C_{17} (as in natural estradiol) to C_{16} (as in estradiol-3,16β) results in an almost total loss of estrogenic potency. Nevertheless, the α configuration for the alcohol at C_{16} (as in estradiol-3,16α and in natural estriol) confers considerable estrogenic activity. Like estriol (3), estradiol-3,16α is less active than estrone

[1] These bioassays were performed by the Endocrine Laboratories of Madison, Wisconsin, under the direction of Dr. Elva Shipley.

by assay in the adult, spayed rat, but is more active than estrone in the intact, immature animal.[1]

EXPERIMENTAL[2]

Preparation of Estradiol-3,16α by Epimerization of Estradiol-3, 16β—Pure 1,3,5(10)-estratrien-3,16β-diol (estradiol-3,16β) (2), 1.00 gm., was dissolved in 28 ml. of dry pyridine at ice bath temperature and treated with 4.4 gm. of solid *p*-toluenesulfonyl chloride. After 2 days at room temperature, the solution was diluted with much ice water containing 10 per cent sodium chloride and permitted to remain 24 hours at 5°. The mixture was then extracted with ethyl ether, the ethereal phase being washed four times with equal volumes of water and all aqueous phases being carried through

TABLE I

Comparison of Properties of Estradiol-3,16β and Estradiol-3,16α

Estradiol-3,16	Derivative with C_{16}-α-OH	Derivative with C_{16}-β-OH
Diol	$[\alpha]_D^{25}$ +85° (in 95% ethanol), m.p. 224–224.5°	$[\alpha]_D^{23}$ +74° (in 95% ethanol), m.p. 227–227.5°
Diacetate	$[\alpha]_D^{23}$ +66° (in 95% ethanol), m.p. 117°	$[\alpha]_D^{23}$ +73° (in 95% ethanol), m.p. 96–97°
Dibenzoate	M.p. 130.5–131.5°	M.p. 231–232°
3-Monobenzoate	" 179.5–181°	" 145–146°
3-Methyl ether-16-acetate	" 123–123.5°	" 132–132.5°

a second extraction with ether. The combined ethers were evaporated on the steam bath and the product carefully dried. To the ditosylate (1.90 gm.) in a reaction flask were added 4.8 gm. of freshly fused sodium acetate and 92 ml. of purified acetic acid. The flask, equipped with condenser and calcium chloride protection, was placed in an oil bath and the heat adjusted so that the reaction mixture refluxed briskly (138–150°); refluxing was maintained for 1 hour after solution had become complete. When cool, the steroid was precipitated with ice water and placed at 5° for a day. The product was filtered, dried, and saponified with 0.6 N aqueous methanolic potassium hydroxide under a 1 hour reflux, the methanolic content reduced by distillation, and, after the pH had been adjusted to 5 to 6, the free diol was precipitated with water, filtered, and dried at 40°, yielding 0.88 gm. After recrystallizations from Skellysolve B and from aqueous methanol (charcoal), there was obtained 0.55 gm. of crude estradiol-3,16α melting

[2] All melting points are uncorrected. Microanalyses and optical rotations are by Dr. E. W. D. Huffman, Denver.

at 213–215°. This product was acetylated by using pyridine and acetic anhydride, and the diester recrystallized from aqueous methanol to give 0.63 gm. of estradiol-3,16α diacetate melting at 116–117° (48 per cent). The diacetate was saponified and treated as before (pH 5 to 6), yielding 465 mg. of estradiol-3,16α melting at 223–224°, with decomposition. After recrystallizations from acetone-Skellysolve B and from aqueous methanol (charcoal), a melting point of 224–224.5°, with very slight decomposition, was obtained. A mixed melting point performed with authentic estradiol-3,16β gave a marked depression (195–210°).

$C_{18}H_{24}O_2$. Calculated, C 79.37, H 8.88; found, C 79.40, H 8.96
$[\alpha]_D^{25}$ +85° (c = 0.76 in 95% ethanol)

1,3,5(10)-Estratrien-3,16α-diol Diacetate—1,3,5(10)-Estratrien-3,16α-diol, 52 mg., was acetylated in the usual fashion with acetic anhydride in pyridine. The diacetate, after a recrystallization from 80 per cent methanol, was obtained as short stiff needles melting at 117° (yield, 63 mg.). Subsequent recrystallization did not improve this melting point.

$C_{22}H_{28}O_4$. Calculated, C 74.13, H 7.92; found, C 74.23, H 7.93
$[\alpha]_D^{23}$ +66° (c = 1.09 in 95% ethanol)

3-Benzoxyestra-1,3,5(10)-trien-16α-ol—Estradiol-3,16α, 46 mg., was benzoylated by the Schotten-Baumann method (0.5 N alkali). The monobenzoate was filtered, washed with 0.5 N sodium hydroxide, washed well with water, and dried *in vacuo*. Of crude monobenzoate, a portion (51 mg.) was recrystallized twice from acetone-Skellysolve B and once from aqueous methanol to yield 34 mg. of material melting at 179–181°. An additional recrystallization from 90 per cent methanol gave 26 mg. of balls of tiny needles melting at 179.5–181°.

$C_{25}H_{28}O_3$. Calculated, C 79.75, H 7.50; found, C 79.57, H 7.39

1,3,5(10)-Estratrien-3,16α-diol Dibenzoate—Pure estradiol-3,16α, 150 mg., was dissolved in 6.0 ml. of dry pyridine and the solution treated with 1.5 ml. of benzoyl chloride. The reaction mixture was kept a day at room temperature with occasional swirling, and the dibenzoate then precipitated with ice water. After 24 hours at 5°, the dibenzoate, in the form of a reddish oil, was separated and recrystallized from aqueous ethanolic acetone containing a trace of pyridine. The crystalline material was successively recrystallized, once from aqueous ethanol (charcoal), twice from acetone-Skellysolve B, and once from 95 per cent ethanol to yield 182 mg. of compound melting at 129.5–130.5°. Two further recrystallizations from 95 per cent ethanol gave 132 mg. of very tiny needles melting at 130.5–131.5°.

$C_{32}H_{32}O_4$. Calculated, C 79.97, H 6.71; found, C 80.09, H 6.84

Preparation of 3-Methoxyestra-1,3,5(10)-trien-16α-ol Acetate—3-Methoxyestra-1,3,5(10)-trien-16β-ol (m.p. 103.5–104°) (2) was transformed into the 16-*p*-toluenesulfonate in the usual manner. To the dried product (101 mg.) were added 200 mg. of freshly fused sodium acetate and 4.0 ml. of purified acetic acid, and the epimerization reaction was carried out as previously described. The saponified product was dried and acetylated in pyridine with acetic anhydride in the usual fashion. The acetate was precipitated with ice water. After decantation, the oily compound was dried *in vacuo*, treated with charcoal in acetone, and crystallized from methanol. There resulted 38 mg. of long needles of the estradiol-3-methyl ether-16-acetate, which melted sharply at 123–123.5°.

$C_{21}H_{28}O_3$. Calculated. C 76.79, H 8.59
Found. (*a*) " 76.69, " 8.65
(*b*) " 76.80, " 8.61

Reduction of 16-Ketoestradiol with Sodium Borohydride—1,3,5(10)-Estratrien-3,17β-diol-16-one, 243 mg., dissolved in methanol, was reduced with sodium borohydride at room temperature as usual. The product was thoroughly dried, and the acetonide derivative was then made by dissolving the steroid in 20 ml. of absolute acetone and adding 1.0 ml. of acetone containing hydrogen chloride (N 6.825). The mixture was swirled for 8 minutes. Then, in one portion, 10 ml. of 1 N potassium hydroxide were added, mixed in immediately, and diluted to a volume of 250 ml. with water. After 24 hours at 5°, the precipitated acetonide was filtered and washed well with water containing a trace of pyridine. The dried acetonide was partitioned between 0.1 N sodium hydroxide and chloroform containing a trace of pyridine (4). After separation, the 0.1 N sodium hydroxide phase was reextracted with chloroform. The two chloroform phases were combined, washed with water, and taken to dryness on the steam bath after the addition of a trace of pyridine. The dried acetonide (281 mg.) was then hydrolyzed by refluxing for 30 minutes in a solution of 50 ml. of 95 per cent ethanol plus 10 ml. of 1.2 M phosphoric acid. To the hydrolyzed mixture were added 25 ml. of water, and the alcohol removed by distillation until the solution had become turbid. After a day at 5° the steroid was filtered, washed with water, and dried. The yield was 185 mg., melting at 273–276°. Treatment with charcoal and a recrystallization from aqueous methanol gave 151 mg. of small, glistening, flat needles which melted at 277–278°. On admixture with authentic 16-epiestriol, there was no depression of the melting point.

$C_{18}H_{24}O_3$. Calculated, C 74.97, H 8.39; found, C 75.13, H 8.42
$[\alpha]_D^{25}$ +81° (*c* = 0.62 in dioxane)

3-Methoxyestra-1,3,5(10)-trien-16β,17β-diol Acetonide—16-Ketoestra-

diol, 286 mg., was reduced as usual with sodium borohydride in methanol but with careful temperature maintenance at 20° during the course of 1 hour's reaction time. Of the reduced product, a recrystallized portion (126 mg.) was dissolved in 12.5 ml. of 90 per cent methanol, 1.25 gm. of anhydrous potassium carbonate were added, and then, during a 1 hour interval, methylated with a total of 0.9 ml. of methyl sulfate. The steroid-3-methyl ether was precipitated with 20 ml. of ice water, and then, after a day at 5°, filtered, washed well with water, dried at 80°, and recrystallized from aqueous acetone. However, upon attempted recrystallization from acetone-Skellysolve B, acetonide formation took place. The oily acetonide furnished a crystalline product (110 mg.) from methanol (m.p. 150.5–153°). Another recrystallization from methanol sharpened this melting point to 152.5–153.5° (80 mg.).

$C_{22}H_{30}O_3$.	Calculated.	C 77.15, H 8.83
	Found. (*a*)	" 77.10, " 8.79
	(*b*)	" 76.95, " 8.83

SUMMARY

The preparation of 1,3,5(10)-estratrien-3,16α-diol by the epimerization of estradiol-3,16β is described. Estradiol-3,16α was characterized as the diacetate, the dibenzoate, and 3-monobenzoate, and the 3-methyl ether-16-acetate. Estradiol-3,16α is more dextrorotatory than is estradiol-3,16β.

Discussion and experimental data are presented in support of the spatial assignments at C_{16} of the estradiols-3,16.

The authors wish to thank Dr. Elva Shipley for performing the bioassays cited in this publication. To the Graduate Research Institute of Baylor University (Dallas) the authors are especially indebted for financial assistance rendered during the course of this investigation.

The technical assistance of Mr. Albert Tillotson in a portion of this research is gratefully acknowledged.

BIBLIOGRAPHY

1. Huffman, M. N., and Lott, M. H., *J. Am. Chem. Soc.*, **75,** 4327 (1953).
2. Huffman, M. N., and Lott, M. H., *J. Biol. Chem.*, **213,** 343 (1955).
3. Curtis, J. M., and Doisy, E. A., *J. Biol. Chem.*, **91,** 647 (1931).
4. Huffman, M. N., and Lott, M. H., *J. Am. Chem. Soc.*, **71,** 719 (1949).

16-SUBSTITUTED STEROIDS

XIII. ANDROSTAN-3β,16β-DIOL AND ANDROSTAN-3β,16α-DIOL

By MAX N. HUFFMAN and MARY HARRIET LOTT

(From the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma)

(Received for publication, January 20, 1955)

In 1951 the authors (1) reported the synthesis of androstan-3β-ol-16-one, and showed this androstanolone to be identical with Heard's ketone, a monoketo-3β-hydroxyandrostane isolated in 1939 by Heard and McKay (2) from mare pregnancy urine.

Since the preparation of Heard's ketone, several researchers have requested from the authors samples of the 3,16-diols which correspond to it. Although our work in this field is by no means complete, we wish to describe herewith our research to date in this particular category of 16-oxygenated steroids in order that this knowledge may be in the hands of those engaged in the isolation and identification of natural steroidal products.

In keeping with our previous tentative structural assignments in this group (3), we have designated the diol resulting from the sodium borohydride reduction of androstan-3β-ol-16-one as androstan-3β,16β-diol. By blocking the C_3 carbinol as the benzoate prior to reduction, and by epimerizing the C_{16} carbinol in the *p*-toluenesulfonate-acetate exchange reaction, we have been successful in preparing androstan-3β,16α-diol.

The diacetates of both androstan-3β,16β-diol and androstan-3β,16α-diol are described.

We have as yet made no biological studies with these compounds.

EXPERIMENTAL[1]

Androstan-3β,16β-diol—Androstan-3β-ol-16-one benzoate, 575 mg. (m.p. 208–209°)(2), was dissolved under reflux in 300 ml. of methanol, the solution quickly chilled to room temperature, and then 0.39 gm. of solid sodium borohydride added. After the solution had been swirled for 1 hour, 4 ml. of 50 per cent acetic acid were cautiously added, the solution was reduced to a volume of 100 ml. by distillation at the steam bath, and then treated with 100 ml. of ice water. After 2 days in the ice box there were obtained 550 mg. of *3β-benzoxyandrostan-16β-ol* melting at 168–169°. This melting point was not improved by a recrystallization from 75 per cent methanol.

3β-Benzoxyandrostan-16β-ol, 234 mg. (m.p. 167–167.5°), was saponified

[1] All melting points are uncorrected. Microanalyses and optical rotations are by Dr. E. W. D. Huffman, Denver.

XUM

by refluxing for 1 hour in 2 per cent aqueous ethanolic sodium hydroxide. The cooled solution was partitioned between ethyl ether and dilute sodium hydroxide, and the ethereal phase then washed twice with water and evaporated. A recrystallization from aqueous methanol yielded 152 mg., melting at 175.5–177°. This was recrystallized successively from 70 per cent methanol (charcoal) until the melting point of 179–180° could no longer be improved. The product was dried to constant weight *in vacuo* at 80° over phosphorus pentoxide before analysis.

$C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 78.10, H 11.07

The mother liquors from the recrystallizations cited above were diluted with water, and, after a day at 5°, the steroid was filtered and thoroughly dried. Acetylation was carried out in the usual fashion by using acetic anhydride in pyridine. The *androstan-3β,16β-diol diacetate*, after several recrystallizations from 70 per cent methanol, melted at 96–97° (76 mg. of tiny needles).

$C_{23}H_{36}O_4$. Calculated, C 73.36, H 9.64; found, C 73.42, H 9.72
$[\alpha]_D^{24}$ −20° (*c* = 0.99 in chloroform)

We have prepared this diacetate several times, and believe that we once had in hand a sample which showed a double melting point, one at 96–97° and the other at 120.5–122°. This sample was analyzed correctly for $C_{23}H_{36}O_4$.

Androstan-3β,16α-diol—3β-Benzoxyandrostan-16β-ol, 400 mg. (m.p. 167–167.5°), was dissolved in 8 ml. of dry pyridine at ice bath temperature, and the solution treated with 0.8 gm. of solid *p*-toluenesulfonyl chloride. After a day at room temperature, with occasional swirling, the 16-tosylate was precipitated with ice water, filtered, washed thoroughly with water, and dried *in vacuo*.

The steroid derivative was covered with 1.0 gm. of freshly fused sodium acetate and refluxed for 1 hour in solution with 20 ml. of purified acetic acid (oil bath, temperature 137–153°). After the epimerization mixture had cooled, it was distributed between ethyl ether and water. The ethereal phase was washed with 1 N sodium hydroxide, and then washed twice with water and evaporated to dryness. The ethereal residue was dissolved in a solution of 7.5 gm. of potassium hydroxide, 12.5 ml. of water, and 100 ml. of methanol, and saponified for 24 hours at room temperature. The free diol was extracted with ethyl ether, and the ether washed with 1 N alkali and washed well with water. After evaporation of the ether, the hydrophilic diol (318 mg.) was recrystallized twice from acetone-Skellysolve B, whereupon it no longer showed hydrophilic properties and melted at 184.5–186.5°. Two more recrystallizations gave 101 mg. of material melt-

ing at 186–187°. After two recrystallizations from methyl ethyl ketone–*n*-heptane and two more from acetone-Skellysolve B, the melting point rose to 187.5–188°.

$C_{19}H_{32}O_2$. Calculated. C 78.03, H 11.03
Found. (*a*) " 77.99, " 10.90
(*b*) " 77.92, " 10.96
$[\alpha]_D^{25}$ −4° (*c* = 0.777 in 95% ethanol)

In another epimerization reaction, starting with 508 mg. of 3β-benzoxy-androstan-16β-ol (m.p. 168–169°) and purifying through the diacetate, a yield of 230 mg. of androstan-3β,16α-diol diacetate (m.p. 169–170°) was obtained.

Androstan-3β,16α-diol, 100 mg. (m.p. 187.5–188°), was acetylated at room temperature with acetic anhydride in pyridine. After several recrystallizations from 90 per cent methanol, the *androstan-3β,16α-diol diacetate*, in the form of flat plates, melted at 174–174.5°. This melting point could not be improved.

$C_{23}H_{36}O_4$. Calculated, C 73.36, H 9.64; found, C 73.44, H 9.68
$[\alpha]_D^{23}$ −26° (*c* = 0.963 in chloroform)

SUMMARY

The preparations of androstan-3β,16β-diol and androstan-3β,16α-diol, with their diacetates, are described.

To the Graduate Research Institute of Baylor University (Dallas) the authors are especially indebted for financial assistance rendered during the course of this investigation.

BIBLIOGRAPHY

1. Huffman, M. N., and Lott, M. H., *J. Am. Chem. Soc.*, **73,** 878 (1951).
2. Heard, R. D. H., and McKay, A. F., *J. Biol. Chem.*, **131,** 371 (1939).
3. Huffman, M. N., and Lott, M. H., *J. Biol. Chem.*, **215,** 627 (1955).

COFACTORS OF THE CARBON DIOXIDE EXCHANGE REACTION OF CLOSTRIDIUM BUTYRICUM*

By R. S. WOLFE† and D. J. O'KANE

(From the Laboratory of Microbiology, Department of Botany, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, January 3, 1955)

The reversibility of the phosphoroclastic reaction of *Clostridium butylicum* was demonstrated by Wilson *et al.* (1). They observed the incorporation of $C^{13}O_2$ into the carboxyl group of pyruvate and a much slower incorporation of $CH_3C^{13}OOH$ into pyruvate. The incorporation of CO_2 was accelerated by hydrogen, but no exchange between $HC^{13}OOH$ and pyruvate could be demonstrated.

An essentially similar situation had been found by Utter *et al.* (2) for the phosphoroclastic reaction of *Escherichia coli* in which formate is the rapidly exchanging 1-carbon compound. The failure of acetyl phosphate to exchange in this system was reported by Strecker (3). The cofactor requirements for the formate exchange are known: coenzyme A, cocarboxylase, phosphate, and possibly Mn^{++} (4, 5).

The determination of the cofactor requirements for the *Clostridium* phosphoroclastic reaction (6) has provided an opportunity to study the cofactor requirements of carbon dioxide and acetate exchange in the *Clostridium* system. A brief report of some of these results has appeared (7).

EXPERIMENTAL

Materials—A lyophilized cell-free extract of *Clostridium butyricum* was prepared and stored as described previously. $NaHC^{14}O_3$ was prepared from $BaC^{14}O_3$ obtained from the Atomic Energy Commission, and $CH_3C^{14}OONa$ was purchased from the Nuclear Instrument and Chemical Corporation. The activity of the bicarbonate was determined from barium carbonate plates; sodium acetate was plated directly, and pyruvate was plated as the 2,4-dinitrophenylhydrazone. All counts were made with a Nuclear counter and scaler and are expressed as counts per minute per micromole after appropriate correction for background, self-absorption, and coincidence losses. Sufficient counts were recorded to assure a reliable error of less than 5 per cent. The amount of compound was determined by weighing.

* Aided in part by a grant from the National Science Foundation.

† Present address, Department of Bacteriology, University of Illinois, Urbana, Illinois.

XUM

Warburg flasks for measuring $C^{14}O_2$ or $CH_3C^{14}OONa$ exchange reactions with pyruvate were set up similarly. To one side arm was added 0.1 ml. of M bicarbonate; to the other side arm were added 100 μmoles of pyruvate and sufficient KH_2PO_4 or succinic acid to adjust the pH of the bicarbonate to 6.5 when mixed. The main compartment of each vessel contained 5 to 10 mg. of enzyme preparation, 0.03 M phosphate or succinate buffer at pH 6.5, and water or other additions to a total volume of 3 ml. Labeled acetate was added to the main compartment and unlabeled bicarbonate to the side arm of the vessel to follow the incorporation of acetate into pyruvate; labeled bicarbonate was added to the side arm when the incorporation of $C^{14}O_2$ into pyruvate was followed. The contents of both side arms were tipped at zero time, and the reaction was stopped when desired by acidifying the contents of each flask with 5 N H_2SO_4. Occasionally aliquots were removed prior to acidification for acetyl phosphate determination by the method of Lipmann and Tuttle (8). The contents of the flask were removed, the protein precipitated with trichloroacetic acid, and the pyruvate precipitated as the 2,4-dinitrophenylhydrazone. This precipitate was washed three times with water in experiments involving $C^{14}O_2$ and four times with 0.01 N acetic acid in experiments with $CH_3C^{14}OONa$ and was then lyophilized. The dried precipitate was suspended in petroleum ether and plated onto a weighed aluminum disk for counting.

The coenzyme A (CoA) was obtained from the Pabst Laboratories, and we are indebted to Dr. H. G. Paul for a generous gift of 5-nitro-2-furaldehyde semicarbazone (Furacin).

Rate of Incorporation—The incorporation of $C^{14}O_2$ into pyruvate proved to be very rapid (Fig. 1); with 10 mg. of cell-free extract per flask the exchange was complete within 10 minutes. When 5 mg. of extract were used, the reaction was complete in 40 minutes. The incorporation of $CH_3C^{14}OONa$ in pyruvate was very slow (Fig. 2); in 25 minutes 10 mg. of extract yielded pyruvate containing 6.4 per cent of the label expected if equilibrium had been reached. Labeled acetyl phosphate does not exchange with pyruvate, as would be expected from experiments with *E. coli* (3).

Stability—The cell-free extract was aged by shaking a solution of the extract (20 mg. per ml.) in air at 37° for 1.5 hours. When aliquots of this aged solution were tested for the phosphoroclastic activity, no hydrogen evolution was detectable (Table I). Similar aliquots exchanged $C^{14}O_2$ rapidly, but retained only a small portion of the ability to exchange acetate. The $C^{14}O_2$ exchange reaction is apparently more stable than the acetate exchange or the phosphoroclastic reaction.

Phosphate Requirement—The effect of phosphate on the incorporation of $C^{14}O_2$ into pyruvate is illustrated in Fig. 3. Since it has not been possible to

dialyze the cell-free extract without inactivation, it is presumed that the initial rate of the reaction without added phosphate is due to residual phosphate in the crude cell-free extract. The data indicate that 0.01 M phosphate is sufficient to allow the exchange to proceed at the maximal rate. Phosphate also stimulated the acetate incorporation into pyruvate. With labeled acetate having an activity of 140 c.p.m. per μmole, the specific

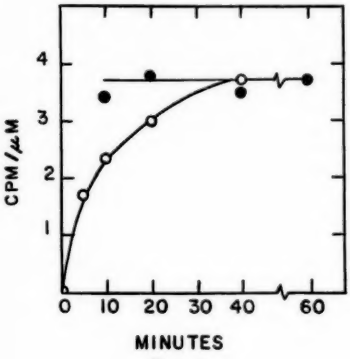


FIG. 1

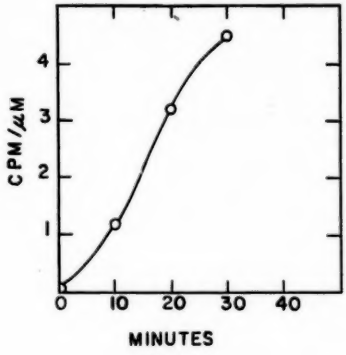


FIG. 2

FIG. 1. Rate of $C^{14}O_2$ exchange. Each flask contained, in a final volume of 3.2 ml., 0.03 M phosphate buffer at pH 6.5, 100 μmoles of potassium pyruvate, 0.1 ml. of M C^{14}-bicarbonate (specific activity 7.7 c.p.m. per μmole), and 0.5 ml. of 0.2 M KH_2PO_4. ●, flasks contained 10 mg. of cell-free extract per flask; ○, flasks contained 5 mg. of cell-free extract per flask. The reaction was stopped at the specified time by acidification with 0.3 ml. of 5 N H_2SO_4. Nitrogen atmosphere.

FIG. 2. Rate of $CH_3C^{14}OONa$ exchange. Each flask contained, in a final volume of 3.2 ml., 10 mg. of cell-free extract, 100 μmoles of potassium pyruvate, 0.1 ml. of M C^{14}-acetate (specific activity, 140 c.p.m. per μmole), 0.1 ml. of M bicarbonate, 0.03 M succinate buffer at pH 6.0, 0.2 ml. of 0.1 M succinic acid, and 0.03 M phosphate at pH 6.5. After tipping the contents of the side arms and equilibration of the CO_2, the pH of the reaction mixture was 6.5. Hydrogen atmosphere.

activity of the pyruvate after a 25 minute reaction was 2.1 c.p.m. per μmole in the absence of added phosphate and 3.2 c.p.m. per μmole in the presence of 0.03 M added phosphate.

Arsenate replaces phosphate in the phosphoroclastic reaction (6) and, as shown in Fig. 4, will replace phosphate in the CO_2 exchange as well. The acetate exchange is markedly stimulated by small amounts of arsenate (0.003 M), but increasing amounts become inhibitory and acetate exchange is negligible at 0.03 M.

Cofactor Requirements—It has been previously reported that the cofactors for the phosphoroclastic reaction of *C. butyricum* include cocarboxy-

TABLE I

Effect of Aging on Phosphoroclastic and Exchange Reactions

Cell-free extract	Hydrogen produced	Activity of pyruvate after incubation with	
		$C^{14}O_2$	$CH_3C^{14}OONa$
	μmoles	*c.p.m. per μmole*	*c.p.m. per μmole*
Untreated	10.3	3.7	3.2
Aged	0.0	3.2	0.8

Flasks for the determination of hydrogen evolution contained, in a final volume of 3.2 ml., 100 μmoles of potassium pyruvate, 0.03 M phosphate at pH 6.5, 5 mg. of cell-free extract, and, in the center well, 0.2 ml. of 20 per cent KOH. Nitrogen atmosphere. Reaction time 20 minutes. The conditions for measuring the exchange reactions were as indicated for Figs. 1 and 2, except that succinate buffer was also present during the determination of carbon dioxide incorporation and 10 mg. of cell-free extract and a 20 minute reaction time were used.

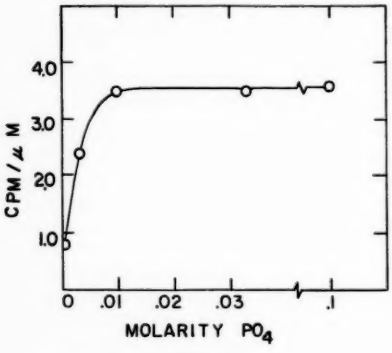


FIG. 3

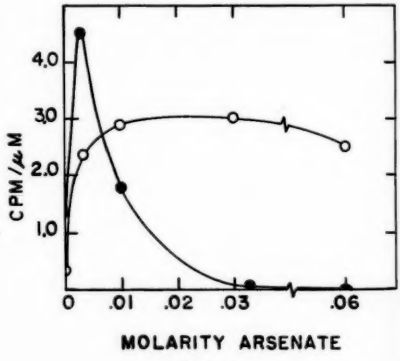


FIG. 4

FIG. 3. Effect of phosphate concentration on the incorporation of $C^{14}O_2$ into pyruvate. Each flask contained, in a final volume of 3.2 ml., 100 μmoles of potassium pyruvate, 0.03 M succinate buffer at pH 6.0, 0.2 ml. of 0.1 M succinic acid, 0.1 ml. of M C^{14}-bicarbonate (specific activity 7.6 c.p.m. per μmole), 5 mg. of cell-free extract, and phosphate as indicated. Reaction time 25 minutes. Nitrogen atmosphere.

FIG. 4. Effect of arsenate concentration on the incorporation of $C^{14}O_2$ and CH_3-$C^{14}OONa$ into pyruvate. ●, acetate exchange. Each flask contains, in a final volume of 3.2 ml., 10 mg. of cell-free extract, 0.1 ml. of M C^{14}-acetate (specific activity 140 c.p.m. per μmole), 0.03 M succinate buffer at pH 6.0, 0.2 ml. of 0.1 M succinic acid, 0.1 ml. of M bicarbonate, and arsenate as indicated. Reaction time 25 minutes. Hydrogen atmosphere. ○, carbon dioxide exchange. Each flask contained, in a final volume of 3.2 ml., 5 mg. of cell-free extract, 100 μmoles of pyruvate, 0.1 ml. of M C^{14}-bicarbonate (specific activity 7.6 c.p.m. per μmole), 0.03 M succinate buffer at pH 6.0, 0.2 ml. of 0.1 M succinic acid, and arsenate as indicated. Reaction time 20 minutes. Nitrogen atmosphere.

lase, coenzyme A, and ferrous ions (6). The cocarboxylase requirement was revealed by alkaline ammonium sulfate precipitation of the cell-free extract, and the coenzyme A and ferrous ion requirements by Dowex treatment of the cell-free extract. Extracts treated by these methods were tested for their ability to catalyze the incorporation of labeled CO_2 or acetate into pyruvate in the presence and absence of the cofactors. These results are presented in Table II and indicate that cocarboxylase and CoA are required for the incorporation of $C^{14}O_2$ into pyruvate, but that ferrous ions have little or no effect on the reaction. The acetate exchange reaction

TABLE II

Effect of Cocarboxylase and Coenzyme A on Exchange Reactions

Experiment No.	Enzyme	Cofactors added	Activity of pyruvate after incubation with	
			$C^{14}O_2$	$CH_3C^{14}OONa$
			c.p.m. per μmole	*c.p.m. per μmole*
8-2	10 mg., ammonium sulfate-treated	None	0.4	0.0
		Cocarboxylase 50 γ	4.0	0.0
3-11	5 mg., Dowex-treated	None	0.0	0.0
		CoA 15 units, Fe^{++} 200 γ	3.2	0.0
		CoA + Fe^{++}	0.1	0.0
			3.1	0.0
8-3	10 mg., no treatment	None		4.9
		CoA 15 units		7.5

Conditions as in Figs. 1 and 2, except that in Experiment 8-2 the flask also contained 5 units of CoA. Experiment 3-11 was continued for 30 minutes. In Experiment 8-3, succinate buffer was omitted, 2 mg. of potassium ascorbate were added, and the reaction was continued for 25 minutes in an atmosphere of 95 per cent H_2-5 per cent CO_2.

was found to be completely destroyed by either alkaline ammonium sulfate or Dowex treatment of the extract. However, it was possible to observe an increased exchange of labeled acetate with pyruvate when untreated extract with added CoA is employed. The addition of cocarboxylase or ferrous ions to the untreated extract did not stimulate the exchange of acetate. The rôle of these cofactors in the acetate exchange must await a method of treating the crude extract that does not destroy the reaction, although it may be expected that cocarboxylase is involved in the exchange.

Inhibitors—Furacin was found to be an excellent hydrogen acceptor in the phosphoroclastic reaction (6), and, when added to some partially inactive preparations, greatly stimulated the rate of reaction. However, as presented in Table III, the addition of Furacin to the reaction mixtures

XUM

completely inhibited both the CO_2 and the acetate exchange reactions. The use of a hydrogen atmosphere instead of nitrogen had no effect. The phosphoroclastic reaction, as followed by acetyl phosphate determinations, was rapid in each case. It therefore appears that Furacin irreversibly ties

TABLE III

Inhibition of Exchange Reactions by Furacin

Labeled reactant	Atmosphere	Furacin	Acetyl phosphate produced	Activity of pyruvate
			μmoles	*c.p.m. per μmole*
$C^{14}O_2$	N_2	−	5.2	3.5
		+	6.0	0.0
	H_2	−	4.3	3.6
		+	6.1	0.0
$CH_3C^{14}OONa$	N_2	−	11.7	1.4
		+	12.7	0.0
	H_2	−	10.8	2.3
		+	12.7	0.0

Experimental conditions as in Figs. 1 and 2 except as indicated. The experiments on acetate incorporation were carried out with 10 mg. of cell-free extract and those on carbon dioxide incorporation with 5 mg. When Furacin was used, excess solid was added.

TABLE IV

Inhibition of Phosphoroclastic and Exchange Reactions by Iodoacetate

	$C^{14}O_2$ exchange		$CH_3C^{14}OONa$ exchange	
	Acetyl phosphate produced	Activity of pyruvate	Acetyl phosphate produced	Activity of pyruvate
	μmoles	*c.p.m. per μmole*	*μmoles*	*c.p.m. per μmole*
Cell-free extract	6.1	3.4	9.6	3.2
Same + 0.001 M iodoacetate	1.6	2.9	2.9	1.0
Inhibition, %	73.8	14.6	69.8	68.7

Conditions as in Fig. 3 except as indicated.

up electrons which are received from the forward reaction and which are necessary for the incorporation of CO_2 and acetate into pyruvate by the reverse reaction. Iodoacetate (0.001 M) was found to inhibit the phosphoroclastic reaction about 76 per cent as measured by acetyl phosphate production. In order to get a clue to the site of this inhibition, the effect of iodoacetate on the CO_2 and acetate exchange was determined. These results, presented in Table IV, indicate that iodoacetate has a relatively small effect on the CO_2 exchange reaction, but that the acetate exchange reaction is inhibited to the same extent that the forward reaction is. The

point of action is therefore likely to be at the acetyl level in the phosphoroclastic reaction, after decarboxylation.

DISCUSSION

The cofactor requirements for the phosphoroclastic reaction and carbon dioxide exchange differ only in that the phosphoroclastic reaction requires ferrous ions in addition to coenzyme A, cocarboxylase, and phosphate required by both reactions. This suggests that the ferrous ions function in the forward reaction after carbon dioxide has been liberated, possibly in the production of hydrogen rather than in place of the familiar divalent ions of pyruvate decarboxylation, magnesium and manganese.

The fact that the carbon dioxide exchange survives aging and is not greatly inhibited by iodoacetate, whereas the forward reaction is inhibited under these conditions, indicates that the carbon dioxide-exchanging step occurs early in the reaction sequence. However, the multiple cofactor requirement for carbon dioxide exchange makes it difficult to visualize the forward reaction occurring through a simple, readily reversible decarboxylation, requiring only cocarboxylase and divalent metal, as a primary step. Instead, a much more complex reaction must be considered. It is tempting, but perhaps unwise at the present time, to compare the *Clostridium* system to the formate system of *Escherichia*. Recent work (9) on that system also indicates previously unappreciated complexity.

The mechanism and significance of the small incorporation of acetate by extracts that fail to incorporate acetyl phosphate, a product of the forward reaction, are still unknown.

SUMMARY

Cell-free extracts of *Clostridium butyricum* rapidly incorporate carbon dioxide into pyruvate. This reaction requires as cofactors cocarboxylase, coenzyme A, and phosphate or arsenate. The carbon dioxide exchange reaction is more stable than the forward reaction and is less sensitive to iodoacetate inhibition. A slow, labile acetate incorporation occurs, which is stimulated by phosphate and coenzyme A.

BIBLIOGRAPHY

1. Wilson, J., Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, **42,** 598 (1948).
2. Utter, M. F., Lipmann, F., and Werkman, C. H., *J. Biol. Chem.*, **158,** 521 (1945).
3. Strecker, H. J., *J. Biol. Chem.*, **189,** 815 (1951).
4. Chantrenne, H., and Lipmann, F., *J. Biol. Chem.*, **187,** 757 (1950).
5. Strecker, H. J., Wood, H. G., and Krampitz, L. O., *J. Biol. Chem.*, **182,** 525 (1950).
6. Wolfe, R. S., and O'Kane, D. J., *J. Biol. Chem.*, **205,** 755 (1953).
7. Wolfe, R. S., and O'Kane, D. J., *Bact. Proc.*, 98 (1954).
8. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
9. Novelli, G. D., Gest, H., and Krampitz, L. O., *Federation Proc.*, **13,** 270 (1954); *Bact. Proc.*, 97 (1954).

SYNTHESIS OF ACETATE FROM FORMATE AND CARBON DIOXIDE BY CLOSTRIDIUM THERMOACETICUM*

By KENNETH LENTZ and HARLAND G. WOOD

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio)

(Received for publication, January 6, 1955)

Clostridium thermoaceticum produces a nearly quantitative yield of acetate from glucose (1). Part of this acetate is formed by the reduction of CO_2 which has previously been produced by the oxidation of glucose (2). Wood (3) demonstrated in mass spectrometer studies that approximately 30 per cent of the acetate molecules synthesized by growing cells in the presence of $C^{13}O_2$ were of the species $C^{13}H_3 \cdot C^{13}OOH$. The difference in mass of $C^{13}H_3 \cdot C^{13}OOH$ from $C^{13}H_3 \cdot C^{12}OOH$ and $C^{12}H_3 \cdot C^{13}OOH$ permitted differentiation on a physical basis of these in mixtures of the three species, whereas differentiation on a chemical basis was impossible. The formation of $C^{13}H_3 \cdot C^{13}OOH$ molecules from $C^{13}O_2$ is similar to an autotrophic synthesis, and the mechanism is therefore of great interest.

This paper is concerned with the rôle of formate in the synthesis of acetate from CO_2 in short term resting cell experiments.

EXPERIMENTAL

Preparation of Cell Suspensions—C. thermoaceticum was grown on a medium of the following composition, in gm. per liter: K_2HPO_4 7.0, KH_2PO_4 5.5, $NaHCO_3$ 10.5, $MgSO_4 \cdot 7H_2O$ 0.1, $(NH_4)_2SO_4$ 0.5, sodium thioglycolate 0.5, glucose 12.6, Difco yeast extract 5.0, Bacto tryptone 5.0. Separate solutions of glucose, of phosphate-bicarbonate, and of the remaining components were sterilized and then combined for inoculation after aseptic saturation of each with CO_2. The cells were cultivated for 48 hours under 100 per cent CO_2 at 55°. They were centrifuged on a Sharples supercentrifuge, and the resulting paste was washed twice with 1 volume of a solution containing 0.1 M potassium phosphate, pH 6.8, and 0.05 M glucose[1]

* This investigation was supported in part by contract No. AT(30-1)-1050 with the Atomic Energy Commission and in part by grants from the American Cancer Society and the Elisabeth Severance Prentiss Fund. The isotope used was obtained on allocation from the Atomic Energy Commission. A preliminary report of this work was presented at the April, 1954, meeting of the American Society of Biological Chemists at Atlantic City, New Jersey.

[1] Better activity was obtained when high concentrations of cells were used and if substrate was present during the washing, although it was possible to wash the cells with phosphate buffer, pH 6.8, and add the glucose later. Considerable variation

XUM

under an atmosphere of nitrogen when $C^{14}O_2$ was to be used, or under CO_2 when formate-C^{14} was to be studied.

Fermentation—The cell suspensions were placed in Erlenmeyer flasks which had been gassed previously with the appropriate gas phase and were shaken for 5 to 10 minutes in a bath at 55° ("preliminary fermentation"), and then the $NaHC^{14}O_3$ or $HC^{14}OONa$ was added. After a further 5 to 10 minute period ("fixation fermentation") the fermentation was ended by the addition of sulfuric acid. Generally in experiments with formate-C^{14} unlabeled formate was added to the reaction mixture prior to the preliminary fermentation and before the isotope was added.

Analyses—At the conclusion of the experiment the flask was shaken at room temperature, and the gas was swept with N_2 through an absorption tower containing CO_2-free alkali to obtain the CO_2. This was followed by a 20 minute gassing with tank CO_2 to displace any $C^{14}O_2$ still in solution or in readily exchangeable form. This gas was not collected. The suspension was centrifuged, the cells were washed twice by resuspending in water and recentrifuging, and all supernatant solutions were combined for analysis.

The volatile acids were obtained by 10 volume steam distillation. The formate was separated from the acetate either by chromatographing the mixture of volatile acids on a Celite partition column (4) or by oxidation of the formate with mercuric oxide. In the latter case, formate-C^{12} was added and a second oxidation was performed to remove all traces of formate-C^{14} prior to steam distillation and degradation of the residual acetate. The methyl and carboxyl carbons of acetate were obtained separately either by the method of Phares (5) or by pyrolysis of the barium salt, followed by conversion of the resulting acetone to iodoform and oxidation of the latter by the method of Shreeve *et al.* (6).

All fractions were converted to $BaCO_3$, duplicate samples were counted with an end window counter, and the values were corrected to zero thickness. All samples were counted with 21 or more counts per minute above background.

RESULTS AND DISCUSSION

Short Term CO_2 Fixation—Since previous experiments had been carried out with growing cells over 2 to 8 day periods (3), it was necessary to determine the extent to which resting cell suspensions could fix $C^{14}O_2$ in short periods of exposure. The data in Table I show that $C^{14}O_2$ is rapidly fixed in both carbons of acetate, in agreement with the pattern of labeling found in growing cells. The carboxyl carbon had a higher specific activity than the methyl carbon, as was previously found; this has been attributed to the

was found in the rate of CO_2 fixation, even when glucose was included during the washing.

carboxyl-CO_2 interchange described by Wood (3), which is superimposed on the synthesis of acetate from CO_2. The apparent change in ratio with length of exposure to $C^{14}O_2$ is not significant, but instead illustrates the range of values that have been observed. The average long term ratio of methyl to carboxyl activity is about 0.67.

Formate-CO_2 Interconversion—Because many anaerobic microorganisms are able to reduce CO_2 to formate (7), and in view of the rôle of formate in the formation of the β-carbon of serine (8), the methyl groups of methionine and choline (9) and thymine (10), it seemed appropriate to test formate as an intermediate in the synthesis of acetate from CO_2. If CO_2 were rapidly converted to formate and then formate and CO_2 combined in some manner

TABLE I

Short Term Fixation of CO_2

Experiment No.	1	2	3
Wet weight of cells, %	50	50	25
Reaction mixture, *ml.*	30	68	36
Gas phase, *ml.*	95	182	464
Duration of exposure to C^{14}, *min.*	3	5	9
Initial CO_2,* *mmoles*	0.61	0.67	1.13
" " * *c.p.m. per μmole*	10,900	75,000	1240
Acetate recovered, *mmoles*	1.15	4.66	1.30
" " *c.p.m. per μmole in CH_3—*	5.7	1,083	40.6
" " " " " "			
—COOH	10.8	1,623	45.0
Ratio, $C^{14}H_3$:$C^{14}OOH$	0.52	0.67	0.90

Gas phase, 95 per cent N_2 and 5 per cent CO_2. Reaction mixture, 0.05 M glucose, 0.05 M potassium phosphate, pH 6.8, cells, and $NaHC^{14}O_3$ as follows: Experiment 1, 1.5 ml. of 0.29 M; Experiment 2, 4.7 ml. of 0.07 M; Experiment 3, 2.8 ml. of 0.095 M.

* Includes both gas phase and added bicarbonate.

to form acetate, experiments with labeled CO_2 would result in acetate with isotope in both carbons of the same molecule, as in the mass spectrometer experiments of Wood (3).

In experiments with $C^{14}O_2$ in the presence of unlabeled formate, a rapid exchange of isotope between $C^{14}O_2$ and pools of unlabeled formate was observed (see Table II). Similarly $HC^{14}OONa$ was found to label a small pool of unlabeled CO_2 within a short time, as demonstrated by the data in the last column of Table II.[2] The slightly higher activity of formate compared to CO_2 in Experiments 5, 6, and 7 is not considered to be significant.

[2] Fermentation balances indicate that H_2 is not produced by *C. thermoaceticum* during the metabolism of pyruvate and glucose. In addition, preliminary tests have shown that this organism, in common with other *Clostridia*, does not possess formic hydrogenlyase activity. The observed exchange between HCOOH and CO_2 therefore seems *not* to occur by means of the reversible hydrogenlyase reaction.

Incorporation of Formate-C^{14} in Presence of Large Pool of Unlabeled CO_2—In order to study the effect of adding labeled formate to short term resting cell fermentations, it was necessary to minimize the specific activity of the $C^{14}O_2$ formed from formate by using a large gas phase of 100 per cent CO_2 and by adding bicarbonate to the reaction mixture. The specific activity of the C^{14} incorporated in the $C^{14}O_2$ was thus usually reduced (compare also Experiment 12, Table IV). In Table III the results of such experiments are presented. It is noted that the methyl to carboxyl ratios of isotope were

TABLE II

Interaction of Formate and CO_2

Experiment No.	4	5	6	7	8
Wet weight of cells, %	50	25	25	25	50
Reaction mixture, *ml.*	30	36	36	36	26
Gas phase, *ml.*	95	464	464	464	224
Duration of exposure to C^{14}, *min.*	3	3	6	9	7
Initial CO_2,* *mmoles*	0.61	1.13	1.13	1.13	0.58
" " * *c.p.m. per μmole*	10,900	1240	1240	1240	0
" formate, *mmole*	0.42	0.90	0.90	0.90	0.65
" " *c.p.m. per μmole*	0	0	0	0	3850
Final CO_2, *mmoles*	0.52	1.86	1.75	2.06	0.5†
" " *c.p.m. per μmole*	2,900	416	332	337	361
" formate, *mmoles*	0.80	1.31	1.29	1.40	0.88
" " *c.p.m. per μmole*	1,900	472	383	354	925

Gas phase, 95 per cent N_2 and 5 per cent CO_2. Reaction mixture, Experiment 4, 0.05 M glucose, 0.1 M potassium phosphate, pH 6.8, 0.013 M $NaHC^{14}O_3$, and cells (formate not present during preliminary fermentation); Experiments 5, 6, and 7, 0.05 M glucose, 0.05 M potassium phosphate, pH 6.8, 0.007 M $NaHC^{14}O_3$, 0.025 M sodium formate, and cells; Experiment 8, 0.05 M glucose, 0.1 M potassium phosphate, pH 6.8, 0.006 M $NaHC^{12}O_3$, and cells (formate not present during preliminary fermentation).

* Includes both gas phase and added bicarbonate.

† Part of sample lost.

4.45 to 6.81 in these formate-C^{14} experiments, compared to values of 0.52 to 0.90 in the $C^{14}O_2$ fixation experiments in the absence of formate (Table I). In Experiment 9 the methyl specific activity was greater than that of the final CO_2. In Experiment 10 the methyl group was about equal in activity to the final CO_2. In none of the experiments with labeled CO_2 has there been observed a methyl to carboxyl ratio greater than 1. It thus appears that the C^{14} from formate was not entering acetate exclusively as CO_2. It should be noted that an undetermined amount of unlabeled acetate derived from glucose fermented in the preliminary period reduced the specific activity of the acetate formed from C^{14} sources. The data of Table III indicated that formate was a better precursor of the methyl group of

acetate than it was of the carboxyl group. It also appeared that formate might be the exclusive source of the methyl group.

Extent of Carboxyl-CO_2 Exchange in Short Term Experiments—Wood (3) showed that acetate is not an inert end-product of the *C. thermoaceticum* fermentation; when $CH_3 \cdot C^{14}OOH$ was added to growing cell fermentations of glucose, 56 per cent of the C^{14} was converted into CO_2 without a concurrent *net* breakdown of acetate. Under similar conditions only 14 per cent of the C^{14} of $C^{14}H_3 \cdot COOH$ entered the CO_2 pool. The possibility

TABLE III

Distribution of Formate Carbon in Presence of Large CO_2 Pools

Experiment No.	9	10
Wet weight of cells, %	25	25
Reaction mixture, *ml.*	40	50
Gas phase, *ml.*	210	250
Duration of exposure to C^{14}, *min.*	10	10
Initial formate,* *mmoles*	1.01	1.25
" " *c.p.m. per μmole*	628	500
" CO_2, *mmoles*	10.3	12.4
Final formate, *mmole*	0.02	0.02
" " *c.p.m. per μmole*	21.6†	230†
" CO_2, *mmoles*	9.24	13.4
" " *c.p.m. per μmole*	60.8	16.5
Acetate recovered, *mmoles*	2.27	0.30
" " *c.p.m. per μmole in CH_3—*	70.4	12.0
" " " " " " *—COOH*	15.8	1.8
Ratio, $C^{14}H_3$:$C^{14}OOH$	4.45	6.81

Gas phase, 100 per cent CO_2. Reaction mixture, 0.05 M glucose, 0.05 M potassium phosphate, pH 6.8, 0.025 M sodium formate, 0.063 M sodium bicarbonate, and cells.

* Includes formate in reaction mixture plus formate-C^{14} added as follows: Experiment 9, 1.0 ml. of 0.01 M; Experiment 10, 1.1 ml. of 0.07 M.

† Values of questionable significance due to small amount of formate recovered.

therefore existed in the present experiments that formate-C^{14} was actually being converted rapidly to *intracellular* $C^{14}O_2$ which was then utilized for the synthesis of doubly labeled acetate by a route other than through formate. During the first stages when formate was breaking down, highly active CO_2 might be present because of incomplete exchange with the unlabeled extracellular pool of CO_2. If such a postulated synthesis of acetate from intracellular CO_2 derived from formate did occur and if there was subsequent exchange of the acetate carboxyl with the low activity extracellular CO_2, the recovered acetate might be predominantly methyl-labeled, as was found.

This alternative explanation for the type of isotope distribution found after adding formate-C^{14} requires a rapid and extensive carboxyl-CO_2 ex-

change in short term experiments. To determine the extent of this exchange the two parallel experiments of Table IV were set up. To Experiment 11 were added doubly labeled acetate of known isotopic distribution and a pool of unlabeled formate. In Experiment 12 the acetate was unlabeled, but the formate was labeled with C^{14}. Both fermentations were allowed to run 5 minutes after the additions were made. It is evident that in Experiment 11 there was no extensive *specific* dilution of the acetate

TABLE IV

Extent of Carboxyl-CO_2 Exchange in Short Term Experiments

Experiment No.	11	12
Wet weight of cells, %	25	25
Reaction mixture, *ml.*	32	32
Gas phase, *ml.*	468	468
Duration of exposure to C^{14}, *min.*	5	5
Initial CO_2, *mmoles*	19.4	19.4
" formate, *mmole*	0.81	0.81
" " *c.p.m. per μmole*	0	1540
" acetate, *mmole*	0.20	0.22
" " *c.p.m. per μmole in* CH_3—	725	0
" " " " " " —*COOH*	1350	0
Ratio, $C^{14}H_3$:$C^{14}OOH$	0.54	0
Final formate, *mmole*	0.18	0.14
" " *c.p.m. per μmole*	1.3*	36.9*
" CO_2, *mmoles*	18.9	20.6
" " *c.p.m. per μmole*	0.6	35.9
" acetate, *mmoles*	1.38	2.27
" " *c.p.m. per μmole in* CH_3—	52.1	26.7
" " " " " " —*COOH*	81.3	8.6
Ratio, $C^{14}H_3$:$C^{14}OOH$	0.64	3.11

Gas phase, 100 per cent CO_2. Reaction mixture, 0.05 M glucose, 0.05 M potassium phosphate, pH 6.8, 0.025 M sodium formate, 0.063 M sodium bicarbonate, 0.007 M sodium acetate (1,2-C^{14} or C^{12}), and cells.

* Value of questionable significance due to the small amount of formate recovered.

carboxyl by unlabeled carbon from the large CO_2 pool, since the ratio of activities of the methyl to carboxyl carbons changed only slightly, from 0.54 to 0.64. Likewise little $C^{14}O_2$ was formed from acetate during this experimental period. The total activity of the acetate was of course diluted by unlabeled acetate formed from the glucose. The data of Experiment 12 show that a typical distribution of formate-C^{14} occurred, producing acetate with a methyl to carboxyl ratio of 3.11 to 1, under conditions in which carboxyl-CO_2 exchange was insufficient to affect the acetate pattern. Thus it appears that the formation of predominantly methyl-labeled acetate from formate-C^{14} is not the result of a secondary carboxyl-CO_2 exchange in the doubly labeled acetate.

Incorporation of Formate-C^{14} in Presence of Small CO_2 Pools—When the formate-C^{14} experiments were carried out (Experiment 8, Table V) in the presence of a small pool of CO_2, the methyl activity was not higher than the carboxyl activity. These results are considered to be consistent with the idea that the methyl may have been derived from formate and the carboxyl from CO_2, because in this case the CO_2 became highly active and thus the carboxyl group acquired C^{14} in high specific activity from the CO_2.

TABLE V

Distribution of Formate or CO_2 in Presence of Small CO_2 Pools

	Formate		CO_2
	Experiment 8*	Experiment 13†	Experiment 4‡
	Duration of exposure to C^{14}		
	7 min.	3 min.	3 min.
Initial formate, *mmole*	0.65	0.41	0.42
" " *c.p.m. per μmole*	3850	7000	0
" CO_2, *mmole*	0.58	0.56	0.61
" " *c.p.m. per μmole*	0	0	10,900
Final formate, *mmole*	0.88	0.72	0.80
" " *c.p.m. per μmole*	925	2570	1,900
" CO_2, *mmole*	0.5	0.60	0.52
" " *c.p.m. per μmole*	361	596	2,900
" acetate, *mmole*	0.62	1.13	1.25
" " *c.p.m. per μmole in* CH_3—	63.2	3.9	2.2
" " " " " " —*COOH*	73.5	2.9	12.3
Ratio, $C^{14}H_3$:$C^{14}OOH$	0.86	1.32	0.18

Gas phase, 95 per cent N_2 and 5 per cent CO_2.

* Details of fermentation in Table II.

† Details of fermentation same as for Experiment 1, Table I, and Experiment 4, Table II.

‡ Details of fermentation in Table II.

It was hoped that reducing the length of the fixation period might lessen the extent of conversion of the formate to carboxyl carbon via CO_2. The 3 minute fermentations shown in Table V therefore were carried out, and in Experiment 13 acetate was recovered with a higher specific activity in the methyl group than in the carboxyl. However, the activity was low in both the methyl and the carboxyl carbons, showing that very little acetate was synthesized from CO_2 or formate. Because of this poor synthesis, this experiment is of doubtful significance. Under identical conditions the acetate formed from $C^{14}O_2$ in the presence of unlabeled formate (last column, Table V) had more label in the carboxyl than in the methyl. The

lower level of activity in the methyl agrees with the hypothesis that it was synthesized from low activity formate. However, again there appears to have been very little synthesis of acetate from CO_2. It is possible that the small amount of C^{14} that was incorporated in the carboxyl group entered by the exchange reaction with CO_2 and that in this case the methyl

TABLE VI

Effect of Pools of Unlabeled CO_2 and Formate on Isotope Distribution in Acetate Synthesized from $C^{14}O_2$

Experiment No.	14	15	16	17
Wet weight of cells, %	50	50	50	50
Reaction mixture, *ml.*	29	29	29	29
Gas phase, *ml.*	31	31	31	31
Preliminary exposure to C^{14}, *min.*	5	5	5	5
Initial CO_2, *mmoles*	1.15	1.15	1.15	1.15
" " *c.p.m. per μmole*	6600	6600	6600	6600
Added CO_2, *mmole*	0	1.00	0	0
" formate, *mmole*	0	0	1.00	0*
	Continuation of exposure			
	0 min.	5 min.	5 min.	5 min.
Final CO_2, *mmoles*	1.11	1.81	1.18	1.07
" " *c.p.m. per μmole*	3530	2530	2410	3370
" formate, *mmole*	0.03	0.06	0.64	0.08
" " *c.p.m. per μmole*	293†	135†	2170	202†
" acetate, *mmoles*	2.60	3.14	3.28	2.94
" " *c.p.m. per μmole in CH_3—*	118	170	199	202
" " " " " " *—COOH*	184	229	246	250
Ratio, $C^{14}H_3$:$C^{14}OOH$	0.64	0.74	0.81	0.81

Gas phase, 100 per cent CO_2. Reaction medium, 0.05 M glucose, 0.1 M potassium phosphate, pH 6.8, 0.007 M $NaHC^{14}O_3$, and cells.

* Added 1 ml. of water; same volume as that containing solution added to Experiment 15 or 16.

† Values of questionable significance due to small amount of formate recovered.

to carboxyl ratio was more a reflection of the exchange reaction than of the total synthesis from formate and CO_2.

Effect of Added $C^{12}O_2$ or $HC^{12}OONa$ on Acetate Synthesized from $C^{14}O_2$— The following series of experiments was carried out in a third approach to the problem of differentiating CO_2 and formate as precursors of acetate carbon in *C. thermoaceticum* fermentations. Four identical fermentations with $C^{14}O_2$ as the gas phase were run for 5 minutes. One (Experiment 14, Table VI) was then acidified in order to obtain a sampling of the acetate at the end of the initial fixation period. To the second flask (Experiment 15)

was added 1.0 ml. of 1.0 M $NaHCO_3$, to the third (Experiment 16) 1.0 ml. of 1.0 M sodium formate, and to the fourth (Experiment 17) 1.0 ml. of water. These last three fermentations were then continued under the *same* labeled CO_2 phase for 5 minutes more. The distribution of isotope in the acetates from each fermentation was then determined. The methyl to carboxyl ratios in those experiments in which no formate was added were within the usual range for $C^{14}O_2$ fixation experiments (see Table I). It had been expected when unlabeled formate was added in Experiment 16 that it would reduce the activity in the methyl group of any acetate which was formed subsequent to the addition of the formate. However, the isotope ratio was approximately the same as when bicarbonate was added in Experiment 15. Because of the extremely rapid isotope exchange between CO_2 and formate, it seems probable that the formate rapidly became as active as the $C^{14}O_2$ and thus formed labeled instead of unlabeled methyl precursor compounds.

Although it is difficult to design a decisive experiment that will clearly differentiate CO_2 and formate as precursors of acetate carbons in *C. thermoaceticum* fermentations, the data from the various types of experiments taken together indicate that a rapid reduction of CO_2 to formate occurs during the fermentation of glucose. The formate thus formed can enter the methyl position of acetate in preference to CO_2 carbon, while the carboxyl position is more directly derived from CO_2. The carboxyl-CO_2 exchange reaction is believed to occur, but to an extent that does not significantly alter the isotope pattern in the acetate newly synthesized under the conditions used in these short term experiments.

If this interpretation is correct, the formation of the methyl carbon of acetate from formate by *C. thermoaceticum* would be another example of the synthesis from 1-carbon compounds of positions in carboxylic acids other than the carboxyl group. In studies with fungi, Jefferson and Foster (11) found that formate-C^{14} was fixed into the methyl groups of lactate, pyruvate, and ethanol produced from glucose under anaerobic conditions. Stadtman and White (12) have reported the synthesis of both carbons of acetate from formate by an amino acid-fermenting species of *Clostridium*, the ratio of methyl to carboxyl specific activities being 1.2:1. They did not describe what rôle was played by CO_2, which is known to be formed by this organism, and they did not advance an explanation for the asymmetric labeling of acetate. Pine and Barker (13) have found that *Butyribacterium rettgeri* produces acetate and lactate labeled in the methyl groups from formate-C^{14} while metabolizing glucose. However, under the conditions of their experiments formate was rapidly converted to $C^{14}O_2$, and the isotope distribution in both acids was similar to that obtained with $C^{14}O_2$ alone. Formaldehyde-C^{14}, on the contrary, produced acetate with a methyl to carboxyl isotope ratio of 1.64:1 and lactate labeled chiefly in the methyl

and carboxyl groups. Leaver (14) has shown that formaldehyde-C^{14} enters the α and β groups of propionic acid during fermentations of glucose by propionic acid bacteria. Formaldehyde-C^{14} has previously been found by Wood (3) to enter the methyl group of acetate produced by *C. thermoaceticum* while fermenting glucose. Unpublished experiments in these laboratories have shown that formaldehyde is rapidly oxidized to formate under the conditions of the short term experiments described above.

It is to be noted that the present results with *C. thermoaceticum* are consistent with the previous results of Wood (3) using $C^{13}O_2$. The rapid interconversion of CO_2 and formate gives equal isotope concentration in the formate and CO_2; thus the result is the equivalent isotopically of a total synthesis of acetate from CO_2.

SUMMARY

Clostridium thermoaceticum in resting cell suspensions has been shown to fix $C^{14}O_2$ rapidly into both carbons of acetate during short term fermentations of glucose. A very rapid interchange between formate and CO_2 was found to occur. In experiments with pools of both CO_2 and formate in which one of these was labeled with C^{14}, formate was found to be a better precursor of the methyl group of acetate than was CO_2, while the carboxyl group appears to be more directly derived from CO_2.

BIBLIOGRAPHY

1. Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J., and Ritter, G. J., *J. Bact.*, **43,** 701 (1942).
2. Barker, H. A., and Kamen, M. D., *Proc. Nat. Acad. Sc.*, **31,** 219 (1945).
3. Wood, H. G., *J. Biol. Chem.*, **194,** 905 (1952).
4. Swim, H. E., and Krampitz, L. O., *J. Bact.*, **67,** 419 (1954).
5. Phares, E. F., *Arch. Biochem. and Biophys.*, **33,** 173 (1951).
6. Shreeve, W. W., Leaver, F., and Siegel, I., *J. Am. Chem. Soc.*, **74,** 2404 (1952).
7. Gest, H., *Bact. Rev.*, **18,** 43 (1954).
8. Sakami, W., *J. Biol. Chem.*, **176,** 995 (1948).
9. Sakami, W., and Welch, A. D., *J. Biol. Chem.*, **187,** 379 (1950).
10. Elwyn, D., and Sprinson, D. B., *J. Am. Chem. Soc.*, **72,** 3317 (1950).
11. Jefferson, W. E., and Foster, J. W., *J. Bact.*, **65,** 587 (1953).
12. Stadtman, T. C., and White, F. H., Jr., *J. Bact.*, **67,** 651 (1954).
13. Pine, L., and Barker, H. A., *J. Bact.*, **68,** 216 (1954).
14. Leaver, F. W., *J. Am. Chem. Soc.*, **72,** 5326 (1950).

A PHOTOMETRIC METHOD FOR THE DETERMINATION OF PROLINE*

By WALTER TROLL† and JOHN LINDSLEY

(From the May Institute for Medical Research of the Jewish Hospital Association, Cincinnati, Ohio)

(Received for publication, October 21, 1954)

The characteristic red color produced when proline interacts with ninhydrin in acidic solutions has recently been proposed for use in the quantitative assay of this imino acid (1, 2). These methods could not, however, be applied directly to the estimation of proline in biological fluids, since other amino acids interfere to some extent and must be removed either by chromatography (1) or by destruction with nitrous acid (2). In the present method, which is a modification of the procedure of Chinard (1), the interfering basic amino acids lysine, hydroxylysine, and ornithine are removed by shaking the solutions with Permutit. Under these circumstances, the method becomes entirely specific for proline when applied to protein hydrolysates, urine, or plasma.

The reaction product of proline in this acidic ninhydrin reaction has been assumed to be the compound isolated by Grassmann and von Arnim (3, 4) from a neutral reaction mixture (1, 2). The distinctly different absorption spectra of the acid (1) and neutral ninhydrin condensation products (5) required elucidation. The acid condensation product has been isolated and appears to have properties of a tautomer of the compound formed in the neutral condensation (3, 4).

EXPERIMENTAL

Proline Method—The following procedure was developed for the determination of proline. The solutions (1 to 5 $\times$ 10^{-5} M proline) were shaken with approximately one-tenth their weight of Permutit[1] for 5 minutes. 5 ml. of this solution, 5 ml. of glacial acetic acid, and 5 ml. of ninhydrin reagent[2] are heated in a water bath for 1 hour in test-tubes with plastic screw caps. The solutions are cooled to room temperature and extracted with 5 ml. of benzene by shaking them vigorously for 5 minutes.

* This study was supported by a grant from the United States Public Health Service.

† Present address, Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts.

[1] Permutit "according to Folin" obtained commercially.

[2] 125 mg. of ninhydrin per 3 ml. of glacial acetic acid and 2 ml. of 6 M phosphoric acid dissolved with heating to 70°. This reagent was stable for at least 24 hours.

The phases are allowed to separate, the benzene phase is transferred to a colorimeter tube, and the optical density determined at 515 mμ.[3]

The Permutit treatment removes lysine and ornithine quantitatively from solutions within the pH range of 1 to 7, while removing no proline (Table I). Cysteine is the only neutral amino acid shown to react in the Chinard method (1), but appears to be absent in the biological materials tested (2). Satisfactory recovery of proline was obtained when added to protein hydrolysates, urine, and plasma (Table I), and the absorption spectrum obtained when these biological materials were subjected to the procedure was identical with that obtained when proline alone was used. Urine was refluxed with 6 M phosphoric acid for 24 hours to liberate proline from conjugates or peptides. Without hydrolysis, urine gave one-third to one-half the proline values obtained after hydrolysis. The initial value,

TABLE I

Proline Recovery

Substance added	Proline added	Proline recovered	
	γ	γ	*per cent*
40 γ bovine serum albumin hydrolysate	2.30	2.25	97.8
	3.35	3.38	100.9
Hydrolyzed urine diluted 20-fold	2.30	2.18	94.8
	3.35	3.40	101.5
Deproteinized plasma diluted 1:14	3.25	3.25	100.0
	3.00	3.08	102.7

however, does not represent free proline, since some hydrolysis of the proline conjugates must take place in the 1 hour reaction period. Unsatisfactory recovery of proline was observed unless urine was diluted at least 20-fold and deproteinized plasma 10-fold with water before assay. The reason for this low recovery appears to be the binding of ninhydrin by other substances such as urea, thereby making it unavailable for reaction with proline. Satisfactory recovery was obtained in situations in which the proline concentration was too low to allow such a dilution by doubling the volume of the reaction system and extracting the color with one-sixth the volume of benzene. Protein hydrolysates were prepared by hydrolyzing 10 mg. of protein in 1 ml. of 6 N hydrochloric acid in a sealed tube at 110° for 24 hours. The protein hydrolysates tested were diluted to 250 ml. before analysis by the method described. The results are summarized in Table II. The standard deviation of the fifteen determinations of proline in protein hydrolysates was computed to be 0.10 per cent.

Observations on Reaction Product—The reaction product of the proline-

[3] Coleman and Beckman spectrophotometers were used in this study.

ninhydrin interaction which occurs in a mixture of acetic and phosphoric acid was prepared as described by Chinard (1). The crude product was recrystallized from a 1:10 mixture of benzyl and ethyl alcohols. An identical product could be obtained by starting the preparation with the yellow intermediate of the neutral condensation product of proline and ninhydrin, mono-(diketohydrindylidene) pyrrole (prepared by adding 1 mole of ninhydrin to 1 mole of proline in ethyl alcohol (3)) and treating the solution

TABLE II
Determination of Proline

Proteins	Observed results*	Other methods
	gm. per 100 gm. protein	*gm. per 100 gm. protein*
Bovine serum albumin† (crystalline)	4.95 ± 0.03	5.07 (6)‡
		5.02 (7)
		4.75 (8)
Casein§	11.2 ± 0.05	10.5 (9)
		11.2 (9)
Bovine Achilles tendon‖	12.8 ± 0.0	13.3 (10)
Horse Achilles tendon¶	12.9 ± 0.1	
Carp ichthyocol**	11.1 ± 0.1	
	mg. per 100 ml.	*mg. per 100 ml.*
Plasma	4.12 ± 0.03	1.5–5.7 (11)
	mg. excreted per day	*mg. excreted per day*
Hydrolyzed urine	69.9 ± 0.2	43–86 (12)

* The ± figures represent the standard deviation obtained on three determinations.

† Purchased from Armour and Company, Chicago.

‡ Bibliographic reference numbers.

§ Hammarsten casein purchased from the Nutritional Biochemicals Corporation, Cleveland.

‖ Prepared by the method of Neuman (10).

¶ Obtained through the courtesy of the Lederle Laboratories Division, American Cyanamid Company.

** Prepared by the method of Schmitt *et al.* (13).

with ninhydrin in the acetic-phosphoric acid mixture. Analyses of this compound for nitrogen were identical with those obtained for the neutral condensation product and agreed with the formulation of a di-(diketohydrindylidene) pyrrole (3, 4). The spectra of the acid and the neutral condensation products are strikingly different. The spectrum of the acid condensation product has a maximum at 515 mμ (millimolar extinction 27.2) (Fig. 1), while that of the neutral condensation product has a maximum at 550 mμ (millimolar extinction 82.3) (5). Another difference between the two compounds is their behavior toward alkali in 50 per cent

methyl Cellosolve-water solutions. Only the acid condensation product turned blue when a drop of saturated sodium carbonate was added, the maximum of the spectrum moving to 630 mμ (Fig. 1). The blue form of this compound, unlike the red form, is insoluble in benzene and soluble in polar solvents.

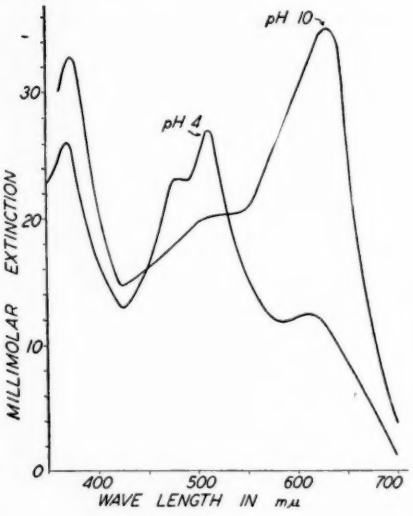


FIG. 1

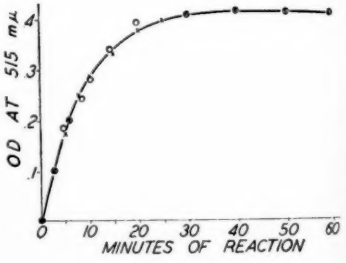


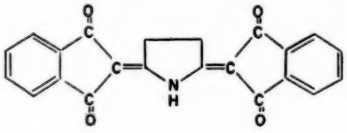
FIG. 2

FIG. 1. Absorption spectrum of the acid condensation product of proline and ninhydrin in 50 per cent methyl Cellosolve-water.

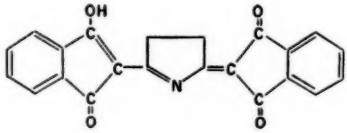
FIG. 2. Rate of formation of the acid condensation product of L-proline and ninhydrin as measured by the optical density at 515 mμ in a Coleman junior spectrophotometer. O, results obtained when 5×10^{-5} M proline was used in the modified Chinard reaction described in the text; ×, results obtained when 5×10^{-5} M mono-(diketohydrindylidene) pyrrole was used as the starting product in the same system.

With the modified Chinard reaction, as described above, the formation of this compound occurs with apparent first order kinetics, and the reaction is complete in about 30 minutes at 100°. It is of interest that the rate of formation of the red condensation product was identical when either proline or the mono-(diketohydrindylidene) pyrrole intermediate was used as the starting product (Fig. 2). The yellow mono compound is apparently an intermediate in the reaction, but the condensation of the 2nd ninhydrin molecule is the rate-controlling step. Yellow color formation has been observed before the red color formation in the Chinard procedure (1).

Two tautomers have been proposed as possible structures of the neutral proline-ninhydrin condensation product (3). The keto form (I) has been shown to be the most likely structure for the neutral condensation product (4). The acid condensation product appears to have the properties of the enol form (II) in that the formation of a polar compound together with a



I



II

striking color change (Fig. 1) would be expected from the ionization of a phenolic type hydroxyl group. No simple explanation is apparent for the complete lack of color formation from hydroxyproline in this system. We have tested the survival of hydroxyproline in the acid ninhydrin system by analyzing for hydroxyproline by a method specific for this imino acid (5) at several intervals during the reaction and have observed that it was rapidly destroyed. This suggests that hydroxyproline reacts with ninhydrin in this system, but that the colored compounds corresponding in structure to proline are too unstable to survive.

SUMMARY

1. A specific photometric method for proline and its application to protein hydrolysates, urine, and plasma has been described.

2. The colored reaction product of proline and ninhydrin formed in acidic solution is not identical with the neutral condensation product which is known to be a di-(diketohydrindylidene) pyrrole. Evidence is presented in support of the view that the acid condensation product is an enol tautomer of this compound.

BIBLIOGRAPHY

1. Chinard, F. P., *J. Biol. Chem.*, **199,** 91 (1952).
2. Schweet, R. S., *J. Biol. Chem.*, **208,** 603 (1954).
3. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **509,** 288 (1934).
4. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **519,** 192 (1935).

5. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, **200,** 803 (1953).
6. Keston, A. S., and Udenfriend, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 92 (1949).
7. Troll, W., *J. Biol. Chem.*, **202,** 479 (1953).
8. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178,** 79 (1949).
9. Brand, E., *Ann. New York Acad. Sc.*, **47,** 213 (1946).
10. Neuman, R. E., *Arch. Biochem.*, **24,** 289 (1949).
11. Harper, H. A., Hutchin, M. E., and Kimmel, J. R., *Proc. Soc. Exp. Biol. and Med.*, **80,** 768 (1952).
12. Stein, W. H., *J. Biol. Chem.*, **201,** 45 (1953).
13. Schmitt, F. O., Gross, J., and Highberger, J. H., *Proc. Nat. Acad. Sc.*, **39,** 459 (1953).

EFFECT OF SUCCINIC AND MALIC ACIDS AND FRUCTOSE ON KETOSIS IN ALLOXAN-DIABETIC RATS*

By CLARISSA H. BEATTY and EDWARD S. WEST

(From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon)

(Received for publication, November 19, 1954)

The current concept of fatty acid metabolism involves the formation of acetyl CoA[1] and the condensation of acetyl CoA with oxalacetate upon entrance into the tricarboxylic acid cycle. Therefore, a sufficient amount of oxalacetic acid is necessary for the complete oxidation of fatty acids via the tricarboxylic acid cycle, and, if sufficient oxalacetic acid is not present, ketosis results.

Previously (1) we have demonstrated that the administration of various precursors of oxalacetic acid, such as succinic and malic acids, and oxalacetic acid itself, decreases total urinary ketone body excretion in non-diabetic rats made ketotic by the administration of butyric acid. This raises the question as to whether or not these substances would have the same effect in an insulin-deficient preparation. Glucose requires insulin to produce oxalacetate at a normal rate, but the effect of insulin on the rate of production of oxalacetate from other precursors is unknown, although recent work (2) indicates that insulin is involved in metabolism at the level of the tricarboxylic acid cycle. There are a few reports in the literature on the effects of succinic acid on ketosis in diabetic humans (3), but the results are conflicting. Therefore, we have investigated the effects of succinic and malic acids on ketosis in insulin-deficient rats. In view of the reports that the utilization of fructose is independent of insulin (4), the effects of fructose on ketosis in these diabetic animals were also investigated.

EXPERIMENTAL

Female Sprague-Dawley rats weighing 200 to 220 gm. were used throughout these experiments. The rats were made diabetic by the subcutaneous injection of 8 mg. per 100 gm. of recrystallized alloxan monohydrate (10 per cent solution). No rats were used less than 4 weeks after alloxan administration, because we have previously shown (5) that ketonuria may be severe directly after the administration of alloxan and some rats do not reach plateau levels of ketonuria in less than 4 weeks. The only rats found

* This investigation was supported in part by research grant No. A-213C from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

[1] Coenzyme A.

to be sufficiently diabetic and yet able to withstand the experiment and remain in good clinical condition were animals with non-fasting blood glucose levels of 450 to 550 mg. per cent. To get dependable ketotic preparations we fasted these diabetic rats 8 hours and at 4.30 p.m. administered 4 to 6 cc. of Wesson oil by stomach tube. The rats were then placed in metabolism cages, and the urine was collected under toluene for a first period of 16 hours. The next morning at 8.30 a.m. another dose of 4 to 6 cc. of Wesson oil was administered and the urine collected for the succeeding 24 hours (second collection period). 2 cc. of Amphojel and 0.2 cc. of a 1:10 dilution of tincture of opium were administered with the oil to prevent diarrhea. Only about 50 per cent of the animals survived the first experiment. However, the rats that proved suitable could usually be used for one experiment per week for several months. These selected animals remained in good clinical condition. While the degree of ketosis established in different rats by the above procedure varied, the level of ketosis for each rat was relatively constant.

Ketone bodies (expressed as acetone) were determined by the method of Greenberg and Lester (6), and glucose was estimated by Somogyi's modification of the Shaffer-Hartmann method (7). The ketone body and glucose excretions in the first urine collection period were determined, and, if either value was out of the control range established for this rat, the experiment was terminated, and the following week the entire experiment was started over again. In this way each rat served as a constant control upon itself. The test solutions were given in five divided doses at 8.30 a.m., 11.00 a.m., 2.00 p.m., 4.30 p.m., and 7.30 p.m. during the second collection period (24 hours). Succinate and malate were given after neutralization to pH 4.5 with NaOH. Therefore, in the control experiments the rats received amounts of Na^+, in NaCl or $NaHCO_3$ solution, equivalent to the maximal amount of Na^+ used. This served as a check on the possible effect of the Na^+ *per se* and on the production of alkalosis with sodium succinate or malate. The amounts of succinate and malate administered were equivalent (on the basis of possible production of oxalacetate) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent or a 20 per cent solution of glucose. Fructose was given as a 20 per cent solution (2 cc. per 100 gm. per 24 hours).

RESULTS AND DISCUSSION

In these experiments no significant difference was found in the ketonuria of rats given NaCl or $NaHCO_3$ solutions ($P > 0.10$ Table I). However, in contrast to the decrease in ketonuria produced by succinate and malate in non-diabetic rats (1), succinate and malate did not decrease ketonuria in these insulin-deficient animals, although the dose administered caused marked glucosuria (Tables II and III). This is in agreement with Bess-

TABLE I

Comparison of Effect of $NaHCO_3$ and NaCl Solutions on Urinary Glucose and Ketone Body Excretion of Alloxan-Diabetic Rats Given Wesson Oil (Second Collection Period)

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

NaCl		$NaHCO_3$	
Ketone bodies	Glucose	Ketone bodies	Glucose
22	0.09	26	0.13
41	0.21	49	0.23
28	0.21	23	0.16
34	0.16	39	0.18
25	0.05	17	0.16
53	0.14	49	0.23
7	0.13	12	0.14
49	0.15	49	0.24
41	0.32	43	0.28

TABLE II

Effect of Insulin, Succinate, and Succinate and Insulin on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

The amount of succinate given was equivalent (on the basis of oxalacetic acid potential) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent glucose solution. Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Succinate		Insulin		Succinate and insulin		Insulin dose, units
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
49–43	0.10–0.17	52	0.40	14	0	2.0	0	1.0
27	0.30	23	0.43					
54–51	0.28–0.18	52	0.62	50	0	21	0.36	0.6
47–41	0.19–0.12	54	0.52	40	0.06	11	0.42	0.7
45–55	0.11–0.12	80*	0.78	49	0.08	20	0.41	0.7
25–23	0.12–0.11	32	0.37	4.0	0.04	0.9	0	1.0
44–45	0.19–0.17	45	0.48	22	0	2.2	0.09	0.8
36	0.16	37	0.40	28	0.04	14	0	0.6
55	0.19			52	0.12	0.7	0.10	0.8
34–42	0.10–0.12	77*	0.60	43	0.09	5.4	0.08	0.7
66	0.36	80*	0.76					
51	0.28	52*	0.62					

* The amount of succinate administered was equivalent, on the basis of oxalacetate potential, to a dose of 2 cc. per 100 gm. per 24 hours of a 20 per cent glucose solution.

XUM

man[2] who gave 5 mmoles of sodium succinate per kilo to human diabetics in coma and observed no change in blood ketones, although the blood sugar increased 90 mg. per cent within 1½ hours. Assuming a two-third distribution of sugar throughout the entire body water, he calculated that this rise would account for complete conversion of succinate to glucose, following the usual path of 2 moles of succinate to 1 mole of glucose. In a few instances the administration of large doses of succinate or malate to

TABLE III

Effect of Insulin, Malate, and Insulin and Malate on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

The amount of malate given was equivalent (on the basis of oxalacetate potential) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent glucose solution. Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Malate		Insulin		Malate and insulin		Insulin dose, units
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
39	0.18	140*	0.78	8	0.09	2	0.11	0.5
		70*	0.56					
22	0.34	22	0.32					
53	0.14	66*	0.40	25	0.07	16	0.35	0.7
61	0.24			42	0.08	4	0.12	0.7
58	0.32			40	0.09	12	0.18	0.6
31	0.18	24	0.52	30	0.09	16	0.12	0.6
26	0.22	44	0.38	20	0.01	12	0.14	0.4
42	0.17	40		40		20	0.33	0.6
43	0.31	43	0.58					

* Dose of malate equivalent, on the basis of oxalacetate potential, to a dose of 2 cc. per 100 gm. per 24 hours of a 20 per cent glucose solution.

our rats appeared to increase ketonuria. This could be explained on the basis of a direct conversion of some succinate to acetate (8) and condensation of 2 acetate molecules to acetoacetate. This ketotic effect of succinate or malate was not observed in non-diabetic ketotic preparations (1).

In order to investigate the rôle insulin may have in succinate metabolism, we determined a dose of insulin which caused no change or a small decrease in the ketonuria of our rats (Tables II and III). When this dose of insulin was administered in conjunction with succinate or malate, the alloxan-diabetic rats showed a much greater decrease in ketonuria than when only insulin was given.

[2] S. P. Bessman, personal communication.

It has been demonstrated that the administration of succinate to humans with diabetes mellitus causes an increase in blood pyruvate concentrations (9) and blood glucose levels.[2] In our own experiments the administration of both malate and succinate caused an increase in glucosuria without affecting the ketonuria. The above evidence indicates that a significant proportion of these test substances entered the usual pathway of metabolism and ran back via oxalacetate through the glycolytic cycle. In six experiments the larger doses of succinate or malate appeared to cause an increase in ketone body excretion. A "ketolytic" effect of succinate and malate could therefore be masked at the lower dosage level. However, we feel that, according to present concepts, a better explanation of the absence of the expected drop in ketonuria may be that utilization of the oxalacetate to decrease ketonuria does not occur in the absence of insulin, suggesting an interference in the condensation of acetyl CoA and oxalacetate.

Since a large part of succinate and malate is converted to glucose, thereby providing extra glucose, it is difficult to distinguish between an insulin effect on glucose and one on condensation of acetyl CoA with oxalacetate. However, if our fundamental assumption is correct that condensation of oxalacetate with acetyl CoA is necessary to prevent ketosis, these experiments appear to indicate that the oxalacetate from succinate and malate is less efficient for condensation in the absence of insulin than in its presence. It has been demonstrated (1) in normal animals made ketotic with butyrate that glucose, succinate, and malate are equally effective, on the basis of oxalacetate potential, in reducing ketosis.

Recent interest in fructose metabolism in diabetes led us to make a comparison of the effects of glucose and fructose on ketonuria in insulin-deficient animals fed Wesson oil. Because some fructose may be converted to glucose by the gastrointestinal tract (10), and because Cori (11) has shown that the gastrointestinal tract absorbs glucose and fructose at different rates, animals were given the sugars intraperitoneally, subcutaneously, and by mouth. The route of injection did not change the effect, probably because the material was given in small doses (five divided doses).

The doses of glucose and fructose used were often larger, on the basis of oxalacetate potential, than the doses of succinate and malate. This was necessary in order to compare the antiketogenic effects of these two substances.

Fructose is removed from the blood stream of diabetics more rapidly than glucose, fructokinase activity being independent of insulin (4). However, this fructose is then metabolized through anaerobic glycolysis. If there is a block in insulin-deficient preparations at the level of the tricarboxylic acid cycle, fructose should prove no more effective than glucose in decreasing ketosis, unless the concentration of fructose is increased suffi-

ciently to exert a mass effect following phosphorylation. Under the conditions of our experiments no difference could be detected between the effects of glucose and fructose on ketonuria (Table IV). Whittlesey and Zubrod (12) observed that fructose was a better ketolytic agent than glu-

TABLE IV

Effect of Glucose and Fructose on 24 Hour Urinary Excretion of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Glucose*		Fructose*		Route of administration
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
51–66	0.28–0.36	35	0.56	71	0.50	Oral
25	0.15	14	0.33	8	0.43	"
55	0.11	4	0.52	17	0.52	"
25–23	0.12–0.11	2	0.48	2	0.48	"
31	0.14	21†	0.71	18†	0.64	Intraperitoneal
42		18†	0.70	14†	0.73	Oral
34	0.09	16‡	0.37	16‡	0.36	Subcutaneous
51	0.18	29	0.58	29	0.86	"
34–33	0.16–0.18	9	0.39	11	0.32	"
38	0.09	3‡	0.29	5‡	0.30	"
34	0.09	3	0.43	2	0.39	"

* 2 cc. per 100 gm. per 24 hours of a 20 per cent solution.

† Repeat determinations on the same rat.

‡ 2 cc. per 100 gm. per 24 hours of a 13.5 per cent solution.

TABLE V

Effect of Fluoroacetate (0.25 Mg. per 100 Gm.) on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Fluoroacetate		Control	
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose
3.1	0.05	35	0.30	1.7	0.14
1.7	0.10	31	0.40	2.1	0.12
2.6	0.18	30	0.18		
2.2	0.03	18	0.20	1.7	
4.3	0.12	8.2	0.25		
1.0	0.07	6.0	0.15		

cose in depancreatized dogs. However, they administered their test substances intravenously, used a much larger dose than in these experiments, and observed their animals for short periods.

We are unable to explain adequately why these larger doses of glucose and fructose decreased ketonuria, while increasing the dose of succinate or malate to a comparable level (on the basis of oxalacetate potential) did not cause a drop in urinary ketone bodies in the absence of insulin.

Previously (1) we demonstrated that sodium fluoroacetate increased urinary ketone body excretion in non-diabetic rats fed butyric acid from 7.4 mg. per 100 gm. per 24 hours to 11.4 mg. per 100 gm. per 24 hours. When the same dose of sodium fluoroacetate was given to alloxan-diabetic rats, the ketonuria increased as much as 18 times (Table V). Fluoroacetate has been shown to block the tricarboxylic acid cycle at the citric acid stage (13).

SUMMARY

1. The administration of succinic and malic acids had no detectable effect on the ketonuria of alloxan-diabetic rats fed Wesson oil, although the dose was sufficient to produce an increased glucosuria.

2. The administration of small amounts of insulin plus succinic or malic acid caused a larger decrease in ketonuria, in our insulin-deficient preparation, than when insulin alone was given.

3. No difference was found between the effects of glucose and fructose on ketonuria in diabetic ketotic rats.

4. The tricarboxylic acid cycle inhibitor, fluoroacetate, has a greater effect in insulin-deficient preparations than in control rats.

5. These results are regarded as presumptive evidence that insulin may be involved in metabolism at the level of the tricarboxylic acid cycle, possibly in the condensation of acetyl CoA with oxalacetate.

BIBLIOGRAPHY

1. Beatty, C. H., and West, E. S., *J. Biol. Chem.*, **190,** 603 (1951).
2. Frohman, C. E., Orten, J. M., and Smith, A. H., *J. Biol. Chem.*, **193,** 803 (1951). Frohman, C. E., and Orten, J. M., *Federation Proc.*, **13,** 215 (1954). Goranson, E. S., and Erulkar, S. D., *Arch. Biochem.*, **24,** 40 (1949). Villee, C. A., and Hastings, A. B., *J. Biol. Chem.*, **181,** 131 (1949). Villee, C. A., White, V. K., and Hastings, A. B., *J. Biol. Chem.*, **195,** 287 (1952).
3. MacKay, E. M., Sherrill, J. W., and Barnes, R. H., *J. Clin. Invest.*, **18,** 301 (1939). Koranyi, A., and Szent-Györgyi, A., *Deut. med. Wochschr.*, **63,** 1029 (1937). Brockmüller, X., *Münch. med. Wochschr.*, **85,** 252 (1938). Dunlop, D. M., and Oxon, B. A., *Lancet*, **2,** 738 (1937). Lawrence, R. D., *Lancet*, **2,** 286 (1937). Dibold, H., Frey, L., and Lapp, F. W., *Deut. med. Wochschr.*, **63,** 1505 (1937).
4. Cori, G. T., Ochoa, S., Slein, M. W., and Cori, C. F., *Biochim. et biophys. acta*, **7,** 304 (1951). Mackler, B., and Guest, G. M., *Proc. Soc. Exp. Biol. and Med.*, **83,** 327 (1953). Dolger, H., Kupfer, S., Bookman, J. J., and Carr, J., *J. Clin.*

Invest., **32,** 562 (1953). Miller, M., Drucker, W. R., Owens, J. E., Craig, J. W., and Woodward, H., Jr., *J. Clin. Invest.*, **31,** 115 (1952). Miller, M., Craig, J. W., Woodward, H., Jr., Owens, J. E., and Drucker, W. R., *J. Clin. Invest.*, **31,** 650 (1952). Craig, J. W., Miller, M., Woodward, H., Jr., and Drucker, W. R., *Am. J. Med.*, **14,** 500 (1953). Daughaday, W. H., and Weichselbaum, T. E., *Metabolism*, **2,** 459 (1953).

5. Beatty, C. H., *Federation Proc.*, **9,** 10 (1950).
6. Greenberg, L. E., and Lester, D., *J. Biol. Chem.*, **154,** 177 (1944).
7. Somogyi, M., *J. Biol. Chem.*, **160,** 61, 69 (1945).
8. Topper, Y. J., and Stetten, D., Jr., *J. Biol. Chem.*, **209,** 63 (1954).
9. Miller, M., Bueding, E., Strauch, R. O., Owens, J., and Woodward, H., *Proc. Am. Diabetes Assn.*, **9,** 3 (1949).
10. Darlington, W. A., and Quastel, J. H., *Arch. Biochem. and Biophys.*, **43,** 194 (1953).
11. Cori, C. F., *J. Biol. Chem.*, **66,** 691 (1925).
12. Whittlesey, P., and Zubrod, C. G., *J. Pharmacol. and Exp. Therap.*, **110,** 226 (1954).
13. Elliot, W. B., and Kalnitsky, G., *J. Biol. Chem.*, **186,** 487 (1950).

THE BIOCHEMICAL RESPONSE TO TRAUMA

I. NUCLEOTIDE CHANGES IN TOURNIQUET SHOCK

By WILLIAM J. JORDAN and IRVING GRAY

(From the Department of Biochemistry, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.)

(Received for publication, December 20, 1954)

The changes that occur in the concentration of chemical constituents of plasma and damaged tissue following trauma have been investigated for many years. The biochemical changes following burns (1) and hemorrhage (2), the protein changes under similar types of injury (3), and those in the nitrogenous compounds (4) have been studied in some detail, and Engel (5) has recently reviewed several aspects of these investigations.

One of the striking changes following trauma is an increase in inorganic phosphate and a decrease in ATP[1] in the tissue (6). In an apparent contradiction to this, Green and Stoner (7) have reported that ATP, injected intramuscularly into rats, can produce all the symptoms of ischemic trauma.

The present study was carried out to determine quantitatively ATP changes as well as alterations in any other nucleotides following trauma. Because of the limited and indirect information obtained by chemical and enzymatic analysis of the various nucleotides, ion exchange chromatography was used.

EXPERIMENTAL

200 to 250 gm. rats were anesthetized with intraperitoneal sodium barbital (50 mg. per animal). Rubber band, arterial tourniquets were applied to both hind legs, and after 4 hours the tourniquets were removed. The mortality with this procedure was 70 per cent in 24 hours (over 100 animals).

At the time of tourniquet release or 90 minutes thereafter, the animals were anesthetized with ether, and the heart and leg muscles removed and dropped into a dry ice-acetone mixture. Corresponding tissues from normal rats were obtained in the same manner. The frozen tissues were removed from the dry ice-acetone, blotted, weighed, and crushed in a chilled steel mortar. The crushed tissue was homogenized in a Waring blendor with 2 volumes (20 ml. to 10 gm. of starting tissue) of cold 0.6 N perchloric acid. The mixture was centrifuged in the cold, the supernatant fluid re-

[1] AMP, ADP, and ATP represent adenosine monophosphate, diphosphate, and triphosphate, respectively; IMP, IDP, and ITP, inosine monophosphate, diphosphate, and triphosphate, respectively.

moved, and the mixture again extracted with 1 volume of acid. The combined supernatant fluids were neutralized in the cold with 5 N KOH to pH 6 to 7, phenol red being used as an internal indicator. The neutralized extract was cooled in a dry ice-acetone bath to the point of freezing and the precipitated $KClO_4$ was removed by centrifugation. The supernatant fluid was made up to volume (100 ml. for leg muscle and 25 ml. for heart) with water, and an aliquot (pH adjusted to 8 with NH_4OH) taken for chromatographic analysis.

Quantitative separation of the adenine nucleotides was effected on 2 cm. columns (1 cm. inner diameter) of Dowex 1 in the chloride cycle (8). However, since, in the method of Cohn and Carter (8), IMP would come off the column with ADP, the first eluting solvent (0.003 N HCl) was increased in volume from 100 ml. to 350 ml. Furthermore, in order to detect any IDP,

TABLE I

Adenine Nucleotide Levels in Hearts from Eight Rats

The values are given in micromoles per gm.

	AMP	ADP	ATP	Total
Normal	1.38 ± 0.81*	2.37 ± 0.96*	1.30 ± 0.92*	5.01 ± 0.80*
T_0†	1.56 ± 0.51	2.35 ± 0.89	1.09 ± 0.78	5.36 ± 1.05
T_{90}†	1.40 ± 0.74	2.40 ± 0.67	1.22 ± 0.80	5.03 ± 0.81

* Standard deviation.

† T_0 and T_{90} represent the animals killed at the time of, and 90 minutes after, tourniquet release, respectively.

a 0.05 M NaCl in 0.01 N HCl elution was used in place of the 0.2 M NaCl-0.01 N HCl solvent. The fractions were identified and quantitatively determined by means of their ultraviolet absorption spectra (9).

RESULTS AND DISCUSSION

In Table I are summarized the results of the analysis of hearts from normal and tourniquet-shocked rats at the time of tourniquet release and 90 minutes after release. It is apparent that there is no significant difference between levels of the adenine nucleotides in normal and traumatized animals.

In Table II are summarized the results of the analysis of the hind leg muscles from normal and traumatized animals at the time of tourniquet release and 90 minutes after release. The ATP level after 4 hour application of tourniquet has dropped to around one-tenth of the normal value and, 90 minutes after the release, has increased slightly but not significantly toward the normal. It should be noted that there is an 8 per cent increase

TABLE II

Adenine Nucleotide Levels in Leg Muscle from Eight Rats

The values are given in micromoles per gm.

	AMP	ADP	ATP
Normal	0.64 ± 0.24*	1.29 ± 0.21*	3.80 ± 0.57*
T_0†		1.34 ± 0.23‡	0.31 ± 0.15
T_{90}†	0.48 ± 0.15	0.83 ± 0.11‡	0.57 ± 0.31

* Standard deviation.

† T_0 and T_{90} represent the animals killed at the time of tourniquet release and 90 minutes after release respectively.

‡ From the chromatogram (Fig. 1), it can be seen that the ADP was contaminated by an unidentified substance absorbing at 250 mμ (see the text).

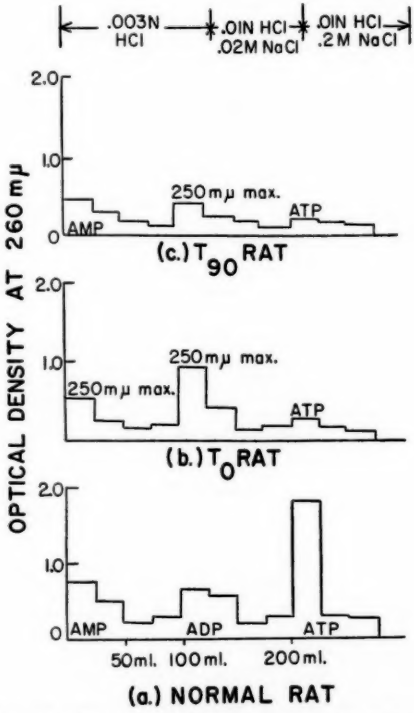


FIG. 1. Separation of adenine nucleotides in extracts of rat leg muscle. The extract represents 1 gm. of tissue. Solutions were applied to the exchanger at pH 8, eluted with H_2O until the eluent became neutral, and then the elution with 0.003 N HCl was started.

in the water content of the T_{90} muscle.[2] However, the values in Table II for the T_{90} rat are not significantly altered by this increase.

The very marked decrease in the level of total ultraviolet-absorbing material apparent in Table II can probably be accounted for in the large

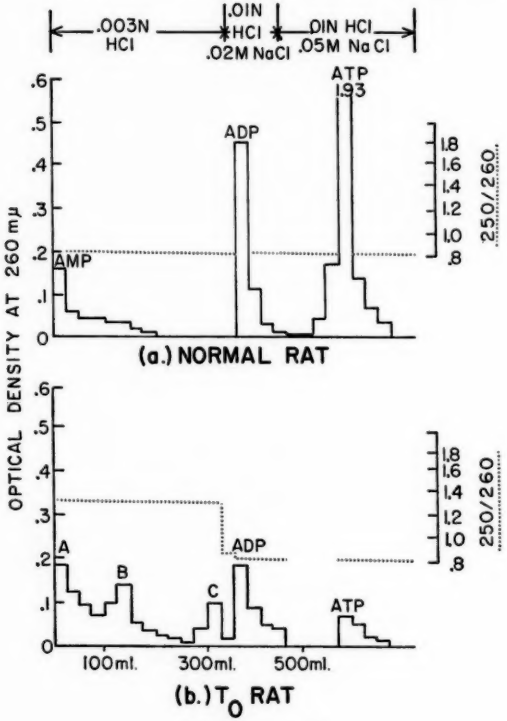


FIG. 2, *a* AND *b*

FIG. 2. *a*, separation of nucleotides in normal rat; *b*, separation of nucleotides in T_0 rat.

increase in ultraviolet-absorbing material found in the preliminary H_2O wash of the T_0 column prior to elution with 0.003 N HCl. The identity of this material is under investigation.

The results (Fig. 1) with ADP are complicated by the fact that, even though the optical density at 260 mμ remains about the same, the absorption maximum has changed to 250 mμ. In order to determine the cause

[2] T_0 and T_{90} represent the animals killed at the time of, and 90 minutes after, tourniquet release, respectively.

of this shift, the elution volume was changed, and it became apparent (Fig. 2, *b*) that this component is made up of three separate peaks, ADP and two unknown peaks, B and C.

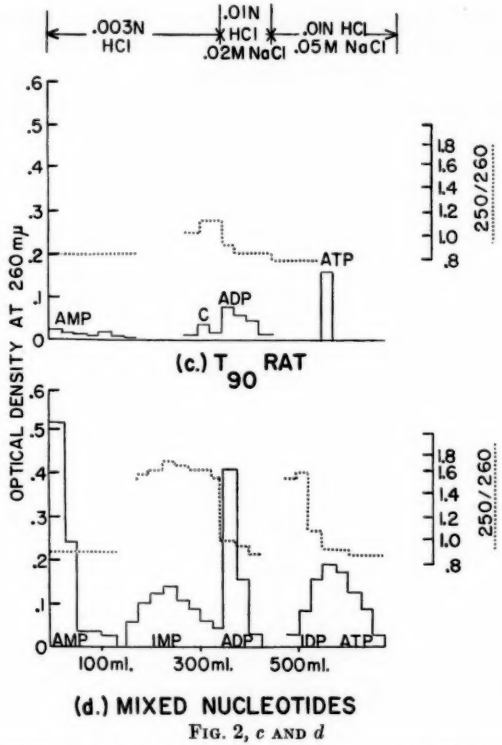


FIG. 2, *c* AND *d*

FIG. 2. *c*, separation of nucleotides in T_{90} rat; *d*, separation of a synthetic mixture of adenine and inosine nucleotides.

The AMP disappeared after 4 hour application of tourniquet but was again present 90 minutes after tourniquet release.

Peak A, eluted at the same point as AMP (Figs. 1, 2, *b*), with an absorption maximum at 250 mμ, was shown by paper chromatography to be hypoxanthine (Fig. 3).

The possibility, as presented by Green and Stoner (7), that the symptoms of ischemic shock are caused by the release of ATP from traumatized tissue is not supported by our data nor by the findings of Bollman and Flock (10) or Goranson *et al.* (11) that acid-labile phosphate in the occluded

legs is almost completely destroyed in 4 hours, and that little or no resynthesis takes place after the tourniquets are removed.

Kovách *et al.* (12) have reported that ATP levels decrease not only in the injured area but also in the heart, brain, and uninjured muscle, whereas Denson and coworkers (13) found an increase in ATP phosphorus in the heart following tourniquet shock. Our data indicate no change in the level of this compound in the heart after application of tourniquet.

It has been shown by Kleinzeller (14), Krebs and Hems (15), and Green, Brown, and Mommaerts (16) that the enzymatic breakdown of ATP in the

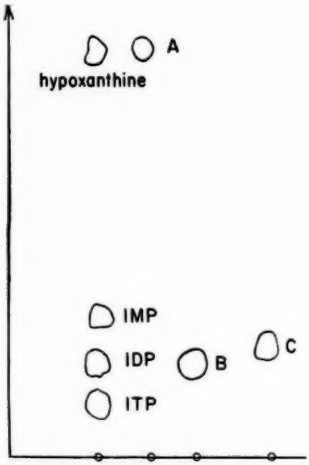


FIG. 3. Chromatogram of Peaks A, B, and C. Solvent, propanol-NH_3-H_2O, 60:30:10, developed for 18 hours. The spots were detected by means of an ultraviolet lamp, Mineralight model SL-2537.

muscle eventually leads to the formation of IMP. Webster (17), on the other hand, detected a direct deamination of ADP to IDP by washed myofibrils. Although we have been unable to detect the presence of IMP in muscle extracts chromatographed immediately after preparation, Peaks B and C (Fig. 2, *b*) were found to have absorption spectra similar to the inosine nucleotides. This is in agreement with the work of Hoffmann, Rottino, and Albaum (18), who detected a substance that also exhibited an absorption spectrum similar to the inosine nucleotides in the blood of several patients in shock.

When moved on paper with Webster's solvent system (17), Peak B coincided with IDP, and Peak C was between IMP and IDP (Fig. 3). However, when moved with the solvent system of Krebs and Hems (15), both

unknowns moved as IMP (Fig. 4). Also, upon standing several days in the refrigerator, both compounds not only moved as IMP in both solvent systems but also are eluted from an ion exchange column predominantly as IMP. From these data, we feel that the two compounds are either unstable isomers of IMP or substances readily degraded to IMP. The possibility exists that unstable, cyclic isomers may be involved. These may be similar to the cyclic precursor reported for adenosine-2- and adenosine-3-phosphoric acids (19) but possibly in a ring from the 5 to 3 or 2 position.

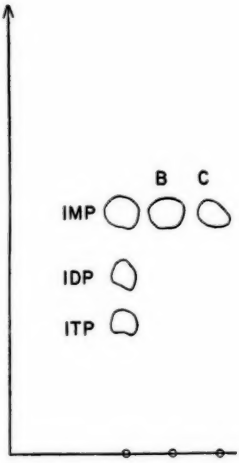


FIG. 4. Chromatogram of Peaks B and C. Solvent, 100 ml. of isobutyric acid (pure solvent), 60 ml. of 1 N NH_4OH, and 1.6 ml. of 0.1 M ethylenediaminetetraacetate, pH 4.4, developed for 18 hours. The spots were detected by means of an ultraviolet lamp, Mineralight model SL-2537.

Further work is being done on the identification of the unknown inosine derivatives, as well as attempts to evaluate their rôle in the shock syndrome.

SUMMARY

ATP has been measured in cardiac and skeletal muscle of normal and tourniquet-shocked rats by ion exchange chromatography.

There was no change in the ATP level in the heart. The level in the occluded muscle dropped to about one-tenth of the normal value, and 90 minutes after the tourniquet release was still depressed.

Two unknown inosine derivatives have been detected in ischemic muscle. These derivatives appear to be unstable, spontaneously changing to IMP. Their structure is discussed.

BIBLIOGRAPHY

1. Lund, C. C., Green, R. W., Taylor, F. H. L., and Levenson, S. M., *Surg., Gynec. and Obst.*, **82,** 443 (1946).
2. Wiggers, C. J., The physiology of shock, New York (1950).
3. Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, **170,** 551 (1947).
4. Walker, J., *Surgery*, **19,** 825 (1946).
5. Engel, F. L., *Ann. New York Acad. Sc.*, **55,** 381 (1952).
6. LePage, G. A., *Am. J. Physiol.*, **146,** 267 (1946).
7. Green, H. W., and Stoner, H. B., Biological action of adenine nucleotides, London (1950).
8. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72,** 4273 (1950).
9. Kalckar, H. M., *J. Biol. Chem.*, **167,** 445 (1947).
10. Bollman, J. L., and Flock, E. V., *Am. J. Physiol.*, **142,** 290 (1944).
11. Goranson, E. S., Hamilton, J. E., and Haist, R. E., *J. Biol. Chem.*, **174,** 1 (1948).
12. Kovách, A. G. B., Bagdy, D., Balázs, R., Antoni, F., Gergely, J., Menyhárt, J., Iránvi, M., and Kovách, E., *Acta physiol. acad. sc. Hung.*, **3,** 331 (1952).
13. Denson, J., Gray, E., and Jensen, H., *Federation Proc.*, **12,** 195 (1953).
14. Kleinzeller, A., *Biochem. J.*, **36,** 729 (1942).
15. Krebs, H. A., and Hems, R., *Biochim. et biophys. acta*, **12,** 172 (1953).
16. Green, I., Brown, J. R. C., and Mommaerts, W. F. H. M., *J. Biol. Chem.*, **205,** 493 (1953).
17. Webster, H. L., *Nature*, **172,** 453 (1953).
18. Hoffmann, G. T., Rottino, A., and Albaum, H., *Science*, **114,** 188 (1951).
19. Brown, D. M., Magrath, D. I., and Todd, A. R., *J. Chem. Soc.*, 2708 (1952).

THE INFLUENCE OF D-GLUCURONOLACTONE ON THE EXCRETION OF L-XYLULOSE BY HUMANS AND GUINEA PIGS*

By OSCAR TOUSTER, RUTH M. HUTCHESON, AND LOUISE RICE

(From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee)

(Received for publication, December 27, 1954)

The excretion of L-xylulose by humans with essential pentosuria suggests that this pentose may be a normal metabolite which these individuals accumulate abnormally as a result of a genetic biochemical defect. This substance has been studied but little in recent years, although other keto sugars, ribulose and sedoheptulose, have been shown to have important metabolic rôles (2, 3). The observation that aminopyrine and other drugs stimulate xylulose excretion by pentosurics led Enklewitz and Lasker (4) to the hypothesis that the glucuronogenic nature of these substances is responsible for their effect on pentose excretion. They tested D-glucuronolactone itself and found that it enhanced xylulose excretion in pentosurics but did not lead to the formation of the pentose in a normal human. It occurred to us that the application of chromatographic methods might make it possible to demonstrate the formation of xylulose by normal subjects and laboratory animals. Such a finding would stimulate investigation of the physiological significance of this pentose.

We have previously reported briefly that normal humans and guinea pigs excrete very small amounts of L-xylulose after ingestion of large doses of D-glucuronolactone and that one "non-pentosuric" subject normally excretes traces of this pentose (5). Xylulose has been found in the urine of some normal guinea pigs as well. This variability among individuals of a species probably reflects small differences in kidney threshold for the substance. It is clear that the ability to produce L-xylulose is not limited to humans with a metabolic defect. The present paper reports the experiments which led to this conclusion.

Influence of Glucuronolactone and Sodium Glucuronate on Xylulose Excretion by Pentosuric Subject

Since glucuronolactone and sodium glucuronate are metabolized differently in the rat (6), these substances were compared as to their ability to

* This study was supported by grants from the National Science Foundation, the Williams-Waterman Fund of the Research Corporation, and Eli Lilly and Company. A preliminary account was presented before the Division of Biological Chemistry of the American Chemical Society, New York, September 17, 1954 (1).

increase xylulose excretion in a pentosuric subject (G. W.).[1] 5 gm. of D-glucuronolactone, or an equivalent amount (6.6 gm.) of sodium D-glucuronate monohydrate (7), were dissolved in 50 ml. of water and administered orally in one dose. 24 hour urine collections were analyzed by the method of Lasker and Enklewitz (8), which is based on the rapid reduction of Benedict's solution (9) at 55°. Actual chromatographic isolation of xylulose as described later demonstrated the reliability of the method.

Fig. 1 shows the pronounced effect of the lactone in stimulating xylulose excretion. The slight influence of the sodium salt may be due to a small amount of the lactone produced. These two forms of glucuronic acid do not rapidly equilibrate *in vivo*, and the sodium salt, unlike the lactone, is

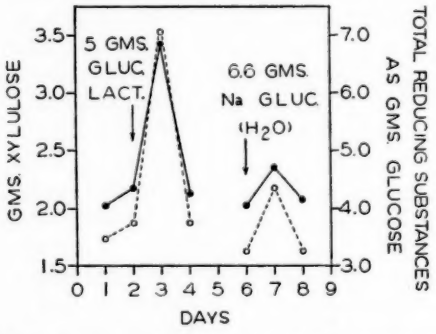


FIG. 1. Effect of oral glucuronolactone and sodium glucuronate monohydrate on xylulose excretion by a subject (G. W.) with essential pentosuria. ●, xylulose, determined by the method of Lasker and Enklewitz (8); ○, total reducing substances, determined by Benedict's procedure (9).

catabolized only to a negligible extent in rats (6). Differences in rate of absorption of the two forms may also have influenced our result. Nevertheless, this experiment indicated that the lactone should be used in efforts to induce xylulose excretion in non-pentosuric subjects.

Experiments on Normal Humans and Guinea Pigs

Collection of Urine—Guinea pigs[2] were maintained in metabolism cages, and the urine of animals of the group under study was pooled until 200 ml. had been collected. In the experiments with glucuronolactone, collection of the urine was commenced simultaneously with the feeding (by pipette

[1] We are greatly indebted to this subject for participating in the experiments and for supplying us with urine from which we isolated the L-xylulose used in this investigation. The pentose was purified via the crystalline *p*-bromophenylhydrazone derivative.

[2] Carworth Farms, Inc., New City, New York.

and drinking water) of 0.1 gm. of glucuronolactone per day to each animal. The samples of normal human urine analyzed were aliquots of 24 hour collections. In the glucuronolactone experiments on humans, each subject ingested 17 gm. in drinking water taken in many divided portions over a period of about 30 hours. The urine samples analyzed were aliquots of a 48 hour collection which was begun at the time of the ingestion of the first portion of test substance. Toluene was always the preservative used.

Isolation of Xylulose from Urine—Urine (200 ml.) was deionized by passing it successively over columns of Amberlite IR-120 (50 ml.) and Duolite A-4 (50 ml.), each column then being washed with a 75 ml. portion of water. The resulting solution was evaporated under vacuum (bath temperature below 60°) to a volume of 5 to 10 ml., made 0.01 M to sodium borate, and poured over a column (20 × 1 cm.) of Dowex 1 in the borate form. The preparation of the resin, development of the column with sodium borate, and removal of sodium borate from eluates were carried out according to the general method of Khym and Zill (10), as employed by Lampen (11). However, 50 ml. of 0.005 M sodium borate were passed through the column after the sample, and then 0.01 M sodium borate was used for elution instead of 0.02 M solution. The eluates were at first analyzed by the orcinol method for pentoses (12), but subsequently it was found much more useful to assay the solutions by the cysteine-carbazole method of Dische and Borenfreund (13). Eluates which yielded, in the latter test, a color with an absorption peak between 540 and 550 mμ were freed of sodium borate, evaporated to a very small volume, and analyzed by paper chromatography; anthrone (14) and naphthoresorcinol (15) reagents served to detect xylulose. Fractions purified by paper chromatography were also tested by spectrophotometric examination (Beckman model DU spectrophotometer) of the colors obtained in the orcinol and cysteine-carbazole procedures.

Results

Fig. 2 illustrates the results of the analysis of four human urine samples by column chromatography. It shows that urine contains a very large amount of cysteine-carbazole-positive material, even when glucuronolactone is not administered. Xylulose, usually found in fractions between 200 and 500 ml., could not be associated with any peak in these curves. Paper chromatography showed that the xylulose-containing fractions also contained considerable amounts of other substances which responded to the cysteine-carbazole test, but these separated from xylulose in the solvents employed. Table I lists the solvents used for paper chromatographic identification of purified fractions. The R_F values for authentic L-xylulose are given, together with the values obtained for the xylulose in the urine of

a normal human. All preparations of human and guinea pig xylulose were checked in several solvents. Ribulose[3] was ruled out on the basis of (*a*) its different mobilities in butanol-pyridine-water (10:3:3) and in 88 per cent phenol, (*b*) its color in the naphthoresorcinol test (xylulose, grey-blue or green-blue; ribulose, blue-green), and (*c*) its more difficult elution from the Dowex 1 (borate) column.

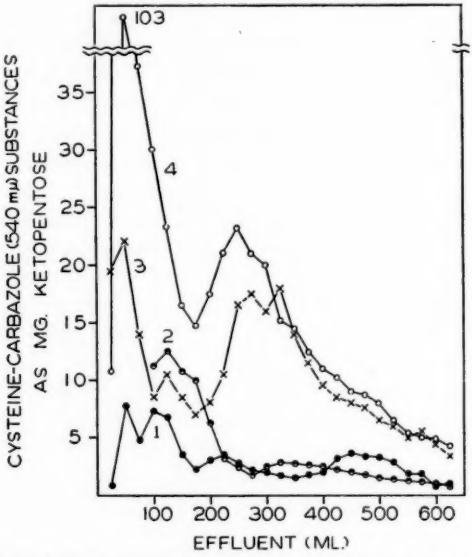


FIG. 2. Fractionation of human urine (200 ml.) on Dowex 1 (borate) columns (see the text for procedure). The effluent was collected in 25 ml. fractions (with a Gilson volumetric fraction collector) and analyzed by the cysteine-carbazole reaction, the color being measured at 540 mμ. Curve 1, subject R. M. after glucuronolactone ingestion; Curve 2, subject R. H. after glucuronolactone; Curve 3, subject O. T. without glucuronolactone; Curve 4, subject O. T. after glucuronolactone.

Table II summarizes our data on the excretion of xylulose by humans. Although only one subject normally excreted the pentose, the three given glucuronolactone responded with an increase in urinary xylulose. The absence of xylulose in the three normal urine samples indicates that the positive results were not due to artifacts. The xylulose found in the normal urine of one subject was eluted from a paper chromatogram, rechromatographed, and again eluted. It yielded the expected absorption spectra

[3] We are indebted to Dr. B. L. Horecker for a sample of D-ribulose *o*-nitrophenylhydrazone.

of the colored products of the orcinol and cysteine-carbazole reactions (see Fig. 3). To demonstrate conclusively that the pentose was L-xylulose this subject was given glucuronolactone (17 gm.) to increase the amount of urinary pentose. This was isolated by the above procedure and purified by repeated paper chromatography until several mg. of product were ob-

TABLE I

Paper Chromatography of Xylulose (R_F Values)

Solvents	L-Xylulose	Normal human urinary xylulose	D-Ribulose
Butyl alcohol-acetic acid-water (4:1:5)	0.42		
Ethyl acetate-pyridine-water (2:1:2)	0.55	0.53	
Amyl acetate-pyridine-water (20:15:5)	0.38		
88% phenol	0.62	0.61	0.69
Butyl alcohol-pyridine-water (10:3:3)	0.42	0.41	0.37
Ethyl acetate-acetic acid-water (9:2:2)	0.40	0.40	0.42
Benzyl alcohol-acetic acid-water (3:1:3), lower layer	0.27		
Isobutyl alcohol	0.20	0.19	

TABLE II

Excretion of Xylulose by Normal Humans

Subject*	Normal xylulose†	Total urinary yield of xylulose from 17 gm. glucuronolactone
	mg. per day	*mg.*
N. K.	<1	
R. H.	<1	72
R. M.	<1	63
O. T.	60	96

* The subjects were between 21 and 33 years old. All except R. H. were male.

† Control experiments showed that 85 to 90 per cent of xylulose added to urine could be recovered by the isolation procedure used. The values in the table are approximate in that they are based upon paper chromatographic estimations.

tained. The light orange oil was treated with 0.014 ml. of phenylhydrazine, 0.09 ml. of water, and 0.01 ml. of glacial acetic acid. The mixture was heated for 3 hours at 90–95°, and ethanol was added to the hot solution until the oily product just dissolved. Upon cooling, the solution yielded a solid product (6.2 mg.) which was recrystallized twice from 40 per cent ethanol. The L-xylosazone melted at 161–162.5° (capillary) and, upon recrystallization in the presence of an equal amount of pure D-xylosazone, yielded the characteristic crystals of DL-xylosazone, m.p. 198–198.5° (capillary) (4).

A total of eleven guinea pigs was studied singly and in groups. Three out of five normal urines analyzed contained detectable amounts of xylulose. Although the stimulatory effect of glucuronolactone was less in guinea pigs than in humans, all five groups of animals fed the lactone yielded urines containing the pentose. The amounts found were 1 to 4 mg. per liter.

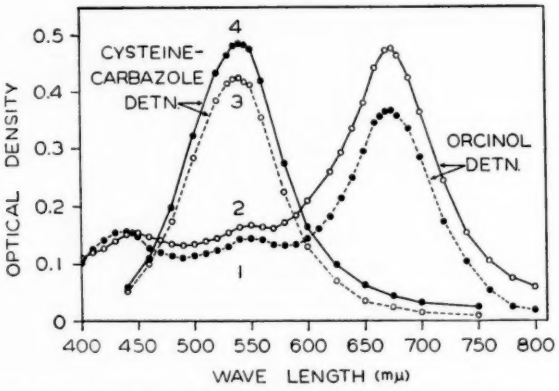


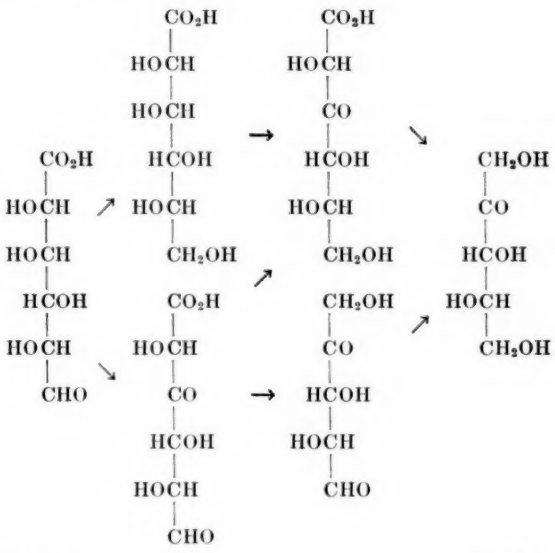
FIG. 3. Absorption spectra of normal human urine xylulose (eluted from a paper chromatogram) and authentic L-xylulose in ketopentose determinations. The reference cell contained a sample obtained by analyzing a control filter paper extract. Curve 1, L-xylulose; Curve 2, isolated xylulose in orcinol test (12); Curve 3, L-xylulose; Curve 4, isolated xylulose in cysteine-carbazole test (13).

DISCUSSION

The failure of previous investigators (4) to find that glucuronolactone yields urinary xylulose in normal humans was probably due to the extremely small amounts of the pentose excreted by these subjects compared to pentosurics, who excrete 1 to 2 gm. of additional xylulose after ingesting only 5 gm. of glucuronolactone. This difference may be due to a very efficient mechanism for the utilization of the pentose by normal humans. This explanation is supported by studies on other mammals in this laboratory. By the procedure outlined in this paper, no xylulose could be detected in the urine of a mouse after intraperitoneal injection of 50 mg. of the pentose (equivalent to 100 gm. in a human). Furthermore, extremely rapid utilization of L-xylulose by slices and homogenates of guinea pig liver has been demonstrated (5, 16).

Recently acquired knowledge of metabolism permits tentative formulation of a mechanism for the transformation of D-glucuronolactone into L-xylulose. The accompanying scheme resembles the biological conver-

sion of D-glucuronolactone to L-ascorbic acid, in which the carbon chain is inverted (17, 18), and utilizes, for the elimination of the carboxyl group of glucuronic acid, reactions similar to those undergone by D-gluconic acid-6-phosphate in its transformation into D-ribulose-5-phosphate (19). It remains to be demonstrated, however, that D-glucuronolactone is directly converted into L-xylulose. The use of labeled glucuronolactone should permit test of this hypothesis.



D-Glucuronic acid (as the lactone)

L-Xylulose

It is interesting that, although the original observation (20) of the stimulatory effect of aminopyrine on xylulose excretion eventually led to the experiments with glucuronolactone, study of the metabolic fate of the drug suggests that its glucuronogenic nature may not be responsible for its effect on urinary xylulose. Over half of a test dose of aminopyrine is excreted as 4-aminoantipyrine and 4-acetylaminoantipyrine (21); yet 2 gm. of the drug lead to the formation of as much xylulose as 5 gm. of glucuronolactone (4). Similarly, 1.3 gm. of antipyrine caused an increase of 1.2 and 2.2 gm. of urinary xylulose in two experiments (4). A study of the mechanism of action of these heterocyclic substances may disclose a hitherto unsuspected effect on enzymatic processes.

SUMMARY

L-Xylulose, the sugar excreted in essential pentosuria, has been found, in trace amounts, in the urine of some guinea pigs and of a normal human. Xylulose was excreted by all three human subjects and all groups of guinea pigs fed large amounts of D-glucuronolactone.

BIBLIOGRAPHY

1. Touster, O., and Hutcheson, R. M., Abstracts, American Chemical Society, 126th meeting, New York, 84C (1954).
2. Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T., and Calvin, M., *J. Am. Chem. Soc.*, **76,** 1760 (1954).
3. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207,** 393 (1954).
4. Enklewitz, M., and Lasker, M., *J. Biol. Chem.*, **110,** 443 (1935).
5. Touster, O., Hutcheson, R. M., and Reynolds, V. H., *J. Am. Chem. Soc.*, **76,** 5005 (1954).
6. Packham, M. A., and Butler, G. C., *J. Biol. Chem.*, **207,** 639 (1954).
7. Hach, W., and Benjamin, D. G., *J. Am. Chem. Soc.*, **76,** 917 (1954).
8. Lasker, M., and Enklewitz, M., *J. Biol. Chem.*, **101,** 289 (1933).
9. Benedict, S. R., *J. Am. Med. Assn.*, **57,** 1193 (1911).
10. Khym, J. X., and Zill, L. P., *J. Am. Chem. Soc.*, **74,** 2090 (1952).
11. Lampen, J. O., *J. Biol. Chem.*, **204,** 999 (1953).
12. Mitsuhashi, S., and Lampen, J. O., *J. Biol. Chem.*, **204,** 1011 (1953).
13. Dische, Z., and Borenfreund, E., *J. Biol. Chem.*, **192,** 583 (1951).
14. Johanson, R., *Nature*, **172,** 956 (1953).
15. Bryson, J. L., and Mitchell, T. J., *Nature*, **167,** 864 (1951).
16. Touster, O., Reynolds, V. H., and Hutcheson, R. M., *Federation Proc.*, **14,** 293 (1955).
17. Isherwood, F. A., Chen, Y. T., and Mapson, L. W., *Biochem. J.*, **56,** 1 (1954).
18. Horowitz, H. H., and King, C. G., *J. Biol. Chem.*, **205,** 815 (1953).
19. Horecker, B. L., Smyrniotis, P. Z., and Seegmiller, J. E., *J. Biol. Chem.*, **193,** 383 (1951).
20. Margolis, J. I., *Am. J. Med. Sc.*, **177,** 348 (1929).
21. Halberkann, J., and Fretwurst, F., *Arq. inst. biol.*, São Paulo, **11,** 149 (1940).

MUCOPOLYSACCHARIDES OF HEART VALVE MUCOPROTEIN*

By WILLIAM P. DEISS† and ARTHUR S. LEON

(From the Department of Medicine, Medical School, and the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin)

(Received for publication, September 23, 1954)

In a previous report from this laboratory a sulfated mucopolysaccharide preparation from bovine heart valve mucoprotein was described (1). On the basis of its similarity to a mucopolysaccharide fraction in cornea described by Meyer and Chaffee (2), it was tentatively classified as a sulfate ester of hyaluronic acid. While this paper was in press, Meyer *et al.* (3) reported further fractionation of corneal mucopolysaccharide into three distinct mucopolysaccharide components: one, a chondroitin sulfate; one, a hyaluronic acid-like fraction; and the third, a new fraction called keratosulfate, containing glucosamine, acetyl, sulfate, and galactose in equimolar concentration.

The purpose of this paper is to describe the further fractionation of the heart valve mucopolysaccharide preparation by means of a scheme similar to Meyer's. Three distinct mucopolysaccharides were obtained, each of which was found to contain a single hexosamine. Two of these were sulfated galactosamine-containing components and the other a non-sulfated glucosamine-containing mucopolysaccharide.

EXPERIMENTAL

Analytical Methods—The analytical methods were those previously employed (1). Uronic acid of Fraction I was determined by decarboxylation by the method of Tracey (4). The separation of the hexosamines was accomplished on a Dowex 50 (hydrogen form) column by a modification of the method of Gardell (5). The modification employed consisted of eluting the hexosamines from the same type and size column used by Gardell, but, instead of 0.3 N HCl as the eluent, the concentration gradient principle of elution (6) was employed. 0.6 N HCl in the reservoir was slowly forced under pressure into a 500 ml. mixing flask, initially filled with water, from which there was an asymptotic increase in the HCl concentration of the eluent forced into the resin column. This modification was found to im-

* Supported in part by a grant from the National Institutes of Health, United States Public Health Service.

† Present address, Departments of Medicine and Biochemistry, Duke University School of Medicine, Durham, North Carolina.

prove the sharpness of the separation and to improve greatly the predictability of the eluate volume at which the hexosamines came off the column. By using a column 35 cm. long and 0.7 cm. in diameter, it was possible to get excellent separation of quantities of hexosamine in the range from 100 to 900 γ. The eluate was collected in 10 ml. fractions in an automatic collector at a rate of 40 ml. per hour, regulated by altering the pressure applied to the reservoir, until about 150 ml. were collected. At this point the pressure was reduced and fractions of 2 ml. were collected at a rate of 4 ml. per hour. The glucosamine peak appeared at 210 ml. of eluate and the galactosamine at 235 ml. For the colorimetric analysis of the fractions appropriate changes in the Na_2CO_3 concentration were made in the reaction with acetylacetone.

Preparation of Crude Heart Valve Mucopolysaccharide—By the isolation procedure previously described (1), which involves extraction with 0.01 N NaOH, approximately 12 gm. (dry weight) of mucoprotein were obtained from about 100 gm. (expressed as dry weight) of freshly dissected bovine heart valves. The mucoprotein clot, formed by adding glacial acetic acid to a concentration of 1 per cent, was stored in several volumes of acetone in the cold. After air-drying and washing with acidified water, deproteinization was accomplished as before (1) by treating the mucoprotein with recrystallized commercial pepsin and trypsin, followed by Sevag's chloroform-amyl alcohol extraction. Other nitrogenous impurities were partially eliminated by adsorption on $Zn(OH)_2$. After dialyzing against running tap water for several days, the mucopolysaccharide preparation was precipitated with 4 volumes of ethanol. Approximately 3 gm. of this material were obtained. This preparation was found to contain 27 per cent hexosamine, of which 52 per cent was glucosamine and 48 per cent galactosamine, indicating that there was probably a mixture of mucopolysaccharides in what we originally tentatively called heart valve hyaluronosulfate.

Further Fractionation of Heart Valve Mucopolysaccharide—This mucopolysaccharide mixture was dissolved in water. Calcium acetate was added to a concentration of 5 per cent, acetic acid added to a concentration of 0.5 N, and ethanol added dropwise as described by Meyer *et al.* (3) in the fractionation of corneal mucopolysaccharide. A fraction precipitated at 20 per cent ethanol and was termed Fraction I. The hexosamine of this fraction was found by column chromatography to be 100 per cent galactosamine. The precipitate at 50 per cent ethanol, Fraction II, was contained in a mixture of hexosamines (40 per cent glucosamine and 60 per cent galactosamine). This fraction was further fractionated from $(NH_4)_2SO_4$ solution with pyridine (7, 8) to yield a thick interfacial precipitate. This precipitate was removed, washed successively with $(NH_4)_2SO_4$-pyridine, 50 per cent ethanol, 95 per cent ethanol, acetone, and finally with ether. After air drying, it was redissolved in water and the $(NH_4)_2SO_4$-pyridine

fractionation repeated. The precipitate was washed as before and dried to give Fraction II-A, in which glucosamine was the only hexosamine present. The $(NH_4)_2SO_4$-pyridine supernatant fluid, Fraction II-B, was dialyzed against water and finally precipitated with 50 per cent ethanol from

TABLE I

*Analytical Data on Heart Valve Mucopolysaccharide Fractions**

	Fraction I		Fraction II-A		Fraction II-B	
	Per cent	Equivalent per equivalent hexosamine	Per cent	Equivalent per equivalent hexosamine	Per cent	Equivalent per equivalent hexosamine
Nitrogen	2.92	1.53†	3.94	1.41†	3.33	1.58†
Glucosamine	<0.1	0	35.81	1.00	<0.1	0
Galactosamine	24.3	1.00	<0.1	0	25.71	1.00
Uronic acid (carbazole)	12.6	0.48	34.88	0.90	23.38	0.84
" " (decarboxylation)	28.0	1.06				
Acetyl	10.58	1.82	13.03	1.51	4.76	0.77
Sulfate	15.43	1.19	<0.1	0	11.15	0.81
Total reducing sugar, %	55.68		78.00		67.1	
Specific rotation, *degrees*	−69.8		−74.7		−22.9	
Ash, %	13.9		0		13.09	
Moisture, %	9.72		6.25		6.56	

* Calculations based on total weight, including ash and moisture.

† These relatively high nitrogen values are assumed to represent some contamination with products of protein hydrolysis.

TABLE II

Enzymatic Hydrolysis of Heart Valve Mucopolysaccharide Fractions by Testicular and Pneumococcal† Hyaluronidase*

The data are presented in per cent of the total reducing sugar liberated.

Fraction No.	Hyaluronidase	Per cent hydrolysis		
		4 hrs.	24 hrs.	48 hrs.
I	Testicular	2.9	3.0	4.4
	Pneumococcal	2.1	2.1	
II-A	Testicular	15	60	
	Pneumococcal	11.1	59.5	
II-B	Testicular	16.0	29.5	
	Pneumococcal	0	0.2	

* Commercial Alidase (Searle).

† Kindly supplied by Dr. C. V. Seastone of the Department of Medical Microbiology, Medical School, University of Wisconsin.

a calcium acetate-acetic acid solution. All of the hexosamine was galactosamine.

From approximately 3 gm. of the original mucopolysaccharide preparation (from 12 gm. of mucin) the following yields were obtained: Fraction I, 0.365 gm.; Fraction II-A, 0.368 gm.; and Fraction II-B, 0.4 gm. After the three fractions were dried and weighed, they were redissolved in water and subjected to electrodialysis at 4° with a potential of 50 to 150 volts for 24 to 36 hours in order to remove all inorganic sulfate. Fractions I and II-B were then precipitated from calcium acetate-acetic acid solution with ethanol, and Fraction II-A was reprecipitated from a 0.5 per cent potassium acetate solution with ethanol. The analytical data on the three fractions are presented in Table I, and the effect of testicular and pneumococcal hyaluronidases on the fractions is shown in Table II.

Results

Fraction I—This mucopolysaccharide differs from the other galactosamine-containing fraction (No. II-B) in several significant respects. It has a considerably higher negative rotation, the carbazole assay for its uronic acid component gives only 50 per cent of the value obtained by decarboxylation, and, lastly, it is resistant to both pneumococcal and testicular hyaluronidases. In these respects this mucopolysaccharide resembles the chondroitin sulfate B isolated by Meyer and Rapport from a number of mesenchymal tissues including heart valves, tendon, aorta, and skin (9). Meyer has previously noted that the carbazole reaction gives a uronic acid value only 50 per cent that found by decarboxylation.[1] The high value for acetyl was obtained on repeated determinations; no satisfactory explanation for this is apparent.

Fraction II-A (Hyaluronic Acid Fraction)—Studies of this fraction indicate that it is hyaluronic acid, similar in properties to that obtained from other sources. It is precipitated from $(NH_4)_2SO_4$ solution by pyridine, contains glucosamine, acetyl, and hexuronic acid, and has a negligible sulfate content. Its optical rotation is similar to that usually reported for hyaluronic acid (9). It is hydrolyzed by both testicular and pneumococcal hyaluronidases. The electrophoretic mobility, -9.8×10^{-5} sq. cm. per volt per second, is the same as that of the hyaluronic acid peak in synovial fluid in our laboratory under similar conditions (0.1 M Veronal buffer, pH 8.6). This peak was markedly reduced after treatment with pneumococcal hyaluronidase.

Fraction II-B (Chondroitin Sulfate Fraction)—This fraction resembles Meyer's chondroitin sulfate C in that it precipitates in 50 per cent ethanol from calcium acetate-acetic acid solution, contains galactosamine, hexuronic acid, acetyl, and sulfate, is hydrolyzed by testicular but not by pneu-

[1] Meyer, K., personal communication.

mococcal hyaluronidase, and has an optical rotation similar to that given by Meyer for chondroitin sulfate C.

DISCUSSION

It is clear that the heart valve mucopolysaccharide preparation originally tentatively classified as a hyaluronic acid sulfate ester is a mixture of at least three distinct mucopolysaccharides. There seems to be little doubt that one of these is hyaluronic acid. This is evidenced by its precipitation from $(NH_4)_2SO_4$ solution with pyridine (hyaluronic acid is the only known tissue mucopolysaccharide that demonstrates this behavior), its composition of approximately equimolar quantities of glucosamine, hexuronic acid, and acetyl, its negligible sulfate content, its optical rotation, and electrophoretic mobility. Hydrolysis by pneumococcal hyaluronidase is a property of only one recognized glucosamine-containing tissue mucopolysaccharide, hyaluronic acid, and the heart valve fraction is readily attacked by this enzyme. A "hyaluronic acid-like fraction" from heart valves was recently mentioned by Meyer *et al.* (3), containing considerable non-dialyzable sulfate, which may or may not be comparable to the fraction reported here.

The two galactosamine-containing mucopolysaccharides, Fractions I and II-B, are comparable to the chondroitin sulfates B and C of Meyer. It is of interest that Fraction I differs significantly from other chondroitin sulfates (A and C) in its resistance to all hyaluronidases, its higher negative optical rotation, and its low uronic acid value by the carbazole reaction.

SUMMARY

Three distinct mucopolysaccharides from bovine heart valve mucoprotein have been characterized on the basis of their chemical analyses, optical rotations, solubilities, purity of hexosamine content, and enzymatic hydrolysis. Two of these are the sulfated galactosamine-containing mucopolysaccharides classified by Meyer as chondroitin sulfates B and C. The third is hyaluronic acid, which has not been demonstrated previously in this tissue.

BIBLIOGRAPHY

1. Deiss, W. P., and Leon, A. S., *J. Biol. Chem.*, **206,** 375 (1954).
2. Meyer, K., and Chaffee, E., *Am. J. Ophth.*, **23,** 1320 (1940).
3. Meyer, K., Linker, A., Davidson, E. A., and Weissmann, B., *J. Biol. Chem.*, **205,** 611 (1953).
4. Tracey, M. V., *Biochem. J.*, **43,** 185 (1948).
5. Gardell, S., *Acta chem. Scand.*, **7,** 207 (1953).
6. Busch, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **196,** 717 (1952).
7. Hadidian, Z., and Pirie, N. W., *Biochem. J.*, **42,** 260 (1948).
8. Jeanloz, R. W., and Forchielli, E., *J. Biol. Chem.*, **186,** 495 (1950).
9. Meyer, K., and Rapport, M. M., *Science*, **113,** 596 (1951).

THE DISTRIBUTION OF DIALKYLFLUOROPHOSPHATASES IN THE TISSUES OF VARIOUS SPECIES

By L. A. MOUNTER, LIEN TIEN H. DIEN, AND ALFRED CHANUTIN

(From the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia)

(Received for publication, December 23, 1954)

Mazur (1) first showed that the sera and tissues of rabbit and man contained enzymes which hydrolyzed dialkyl fluorophosphates. Augustinsson (2) and Mounter *et al.* (3, 4) have demonstrated that enzymes capable of hydrolyzing dialkyl fluorophosphates may have different properties and characteristics. The present investigation was undertaken to study systematically the occurrence of dialkylfluorophosphatases (DFPases) in tissues of the human, rat, cat, guinea pig, pigeon, and turtle.

Methods

Tissues, obtained immediately after killing the animals, were washed with cold physiological saline, blotted on filter paper, and weighed. Human tissues were obtained at autopsy 8 to 16 hours after death. The tissues were homogenized in an all-glass homogenizer with minimal amounts of distilled water, and the homogenate was filtered through muslin to remove most of the insoluble material. The residue was washed several times with small amounts of distilled water, and these washings were added to the original extract. The final dilution in terms of tissue weight varied from 1:4 to 1:10. The solutions were adjusted to pH 7.6 with $NaHCO_3$, and the DFPase activities were determined by the Warburg technique as described previously (1, 3). The substrate was a 10^{-2} M solution of diisopropyl fluorophosphate (DFP). The tissue extract was incubated in the side arm, together with sufficient metal ions to yield a concentration of 10^{-3} M. When the flask was tipped, after incubation, and its contents mixed with the substrate, the final concentration of metal ions was 10^{-4} M. All analyses were corrected for non-enzymatic hydrolysis.

EXPERIMENTAL

The activities of tissues in six species are presented in Table I. In practically all species the highest activities are observed in the kidneys, livers, and intestinal mucosae. Relatively high values are also noted for rat adrenals and pigeon ovaries. These results show that the DFPases of all tissues, with the possible exception of those of the guinea pig, are activated by Mn^{++} and Co^{++} at a final concentration of 10^{-4} M. The degree

XUM

TABLE I

Distribution of DFPase

The results are expressed in microliters of CO_2 per gm. of wet tissue per 30 minutes, and in activity ratios = (activity with 10^{-3} M metal ion in the side arm)/(activity with no added metal ion).

Organ	Rat			Human			Cat			Guinea pig			Pigeon			Turtle		
	Per gm.	Ratios		Per gm.	Ratios		Per gm.	Ratios		Per gm.	Ratios		Per gm.	Ratios		Per gm.	Ratios	
		Mn^{++}	Co^{++}		Mn^{++}	Co^{++}		Mn^{++}	Co^{++}		Mn^{++}	Co^{++}		Mn^{++}	Co^{++}		Mn^{++}	Co^{++}
Kidney	2550	4.3	2.3	7000	1.3	1.3	4,800	1.6	2.0	1300	1.4	0.9	4500	1.5	1.2	2400	3.2	2.0
	2200	3.2	1.6	9500	1.2	1.3	5,000	1.6	1.8	1400	2.1	0.9						
	4500	3.7	1.8	4600	1.4	1.2	5,400	2.0	2.4									
	4400	3.6	1.7				4,800	1.6	2.0									
	5300	3.1	2.0															
Liver	4200	1.9	1.2	5200	1.2	1.1	3,300	1.5	1.6	950	2.1	0.9	2400	1.1	1.0	700	2.7	1.1
	4000	1.3	1.1	4100	1.1	1.0	2,900	1.9	2.0	1300	1.7	0.9						
	5900	1.2	1.0				1,700	1.8	1.7									
	3800	1.6	1.2															
Intestinal mucosa	2300	2.2	1.4							2200	3.1	1.3	540	7.3		1700	4.2	2.8
	3100	2.4	1.6				7,700	1.9	2.0									
	4200	1.7	1.2				6,500	1.8	1.8									
	2100	2.0					11,500	1.2	1.2									
Spleen	2100	4.5	1.9	1700	1.6	1.4	1,700	2.9	3.2									
	1750	5.1	3.3				2,700	2.1	2.2									
	1450	6.6	2.6				2,100	2.9	3.8									
							1,100	2.2	2.1									
Brain	1300	2.6	1.5							520	3.1	0.8	340	4.4	2.9	450	3.3	1.6
	1700	2.8	1.3															
	900	3.0	1.4															
Heart	1450	3.4	1.8							350	3.6		240	2.5	1.4	670	1.5	1.2
	850	3.3	1.9							280	4.0							
Lung	670	4.0	2.2	175	5.0					620	4.0	2.1	200	2.6	1.8	330	3.0	2.0

Heart	1450	3.4	1.8							350	3.6		240	2.5	1.4	670	1.5	1.2
	850	3.3	1.9							280	4.0							
Lung	670	4.0	2.2	175	5.0					620	4.0	2.1	200	2.6	1.8	330	3.0	2.0
	950	3.6	1.9															
	900	2.6	1.3															
Skeletal muscle	445	4.1	3.2															
	550	2.9	2.8															
Testis	2700	2.9	1.4	1100	2.4	3.0	2,000	2.3	2.1	420	3.2	0.7						
	1250	4.1	1.8															
	1650	4.8	2.2															
Bone marrow	2350	3.3	1.6				1,250	5.1	7.0	1020	3.8	3.7						
	1500	3.5	2.8															
Pancreas				1900	1.7	1.5	630	3.3	3.5									
				750	1.1	1.2	1,250	2.4	2.9									
							1,300	1.2	1.3									
							760	2.2	4.0									
Thymus	1800	4.4	1.7															
	1100	6.2	1.8															
	3500	2.6	1.4															
Ovary							330	3.3	7.0	630	4.1	2.3	2700	2.8	2.6			
							350	5.2	7.1									
Adrenal	4500	6		750	1.8	1.3	1,500	5.2	3.9									

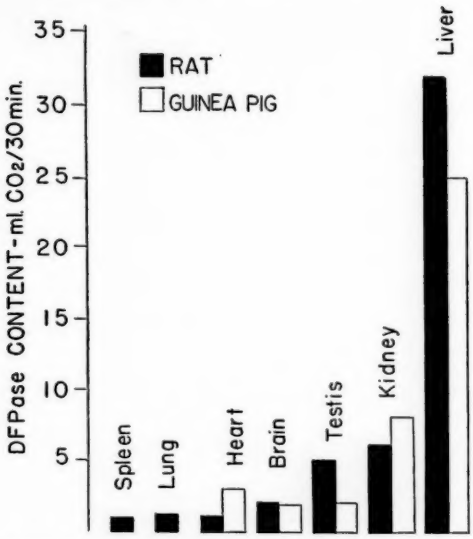


FIG. 1. DFPase content of whole organs of the rat and guinea pig

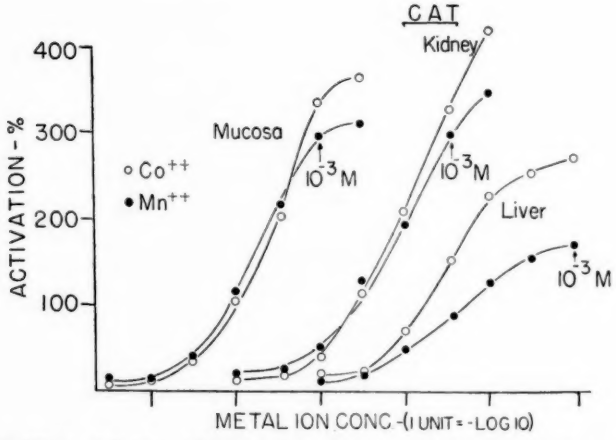


FIG. 2. Effect of Co^{++} and Mn^{++} concentration on DFPase activity in cat tissue. The values for metal ion concentration in this and Figs. 3 to 6 are those in the side arm during incubation.

of activation by metal ions varies considerably. The activities of extracts of one tissue in a single species may also vary over a considerable range.

The DFPase contents of the organs of a rat and a guinea pig are shown in Fig. 1. It is observed that the liver contains more DFPase than the total in the remaining organs studied.

The effect of varying amounts of Mn^{++} and Co^{++} on the activation of extracts of cat, human, and rat tissues is illustrated in Figs. 2, 3, and 4. The DFPases of cat mucosa, kidney, and liver are activated to a greater

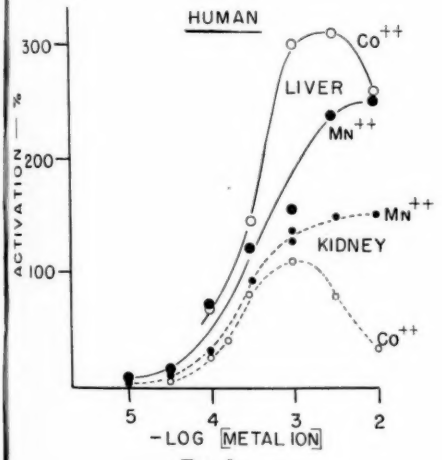


FIG. 3

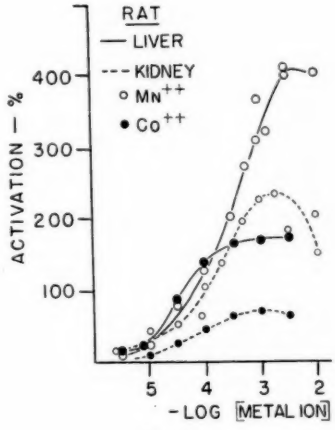


FIG. 4

FIG. 3. Effect of Co^{++} and Mn^{++} concentration on DFPase activity in human tissue.

FIG. 4. Effect of Co^{++} and Mn^{++} concentration on DFPase activity in rat tissue.

extent by Co^{++} than by Mn^{++} at the higher metal ion concentrations. Co^{++} is more effective than Mn^{++} as an activator of human liver DFPase, but is less effective than Mn^{++} with the kidney enzyme. The enzymes in rat kidney and liver are activated more by Mn^{++} than by Co^{++} and in this respect behave similarly to hog kidney DFPase (3). These studies indicate that DFPases of the kidney and liver in different species may have different characteristics. This is comparable to the distinction made between exopeptidases on the basis of metal ion specificity (5).

In contrast to results obtained in studies of hog kidney DFPase, the addition of histidine, 2,2′-dipyridyl, or proline to these tissue extracts does not further activate the enzyme in the presence of Mn^{++} or Co^{++}. These

observations may not be significant, since purified fractions of these extracts were not studied.

In the absence of Mn^{++} there is a direct relationship between the CO_2 evolution and the volume of tissue extract (Fig. 5). Similar experiments in the presence of Mn^{++} show that DFPase activity is potentiated, but a linear relationship is no longer observed. This phenomenon may be due to the binding of metal ions by proteins. The ratios of the respective activities in the presence and absence of Mn^{++}, which are plotted as an activation curve, emphasize the fact that conclusions drawn from studies with tissue extracts have definite limitations.

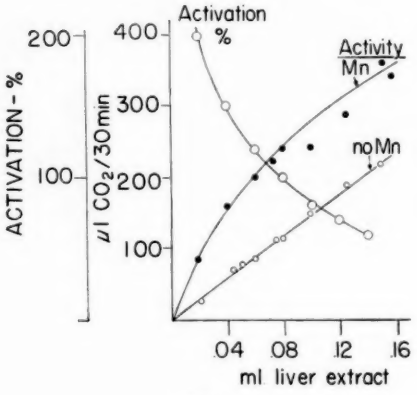


FIG. 5

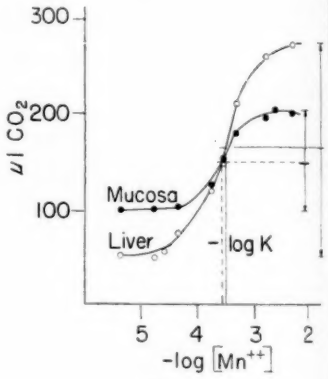


FIG. 6

FIG. 5. Relationship between DFPase activity and volume of rat liver extract.
FIG. 6. Activities and dissociation constants of rat tissue DFPase.

Data for determining the dissociation constants for the DFPases of rat liver and intestinal mucosa extracts are shown in Fig. 6 (6). The values for the dissociation constants of these enzyme-metal complexes (log K) are about 3.6. The values for kidney and testis are 3.5 and 3.6, respectively. The close agreement between constants for the enzymes in four different tissues is in marked contrast to the large variations in activation observed at 10^{-3} M Mn^{++} in Table I. These results emphasize the necessity of working with a constant volume of extract and varying concentrations of metal ion if the influence of metal ions is to be properly interpreted.

The results of this investigation show that DFPases are not only widely distributed in tissues, but may differ from each other in their characteristics. It must be emphasized, however, that the activities of enzymes in tissue extracts are arbitrary values, owing to the variation in protein concentration and naturally occurring activators and inhibitors. Although

the hydrolysis of DFP and its analogues by tissue enzymes cannot be directly correlated with normal metabolic activity, it is probable that the naturally occurring substrates of DFPases possess similar spatial configurations to those of alkyl fluorophosphates.

SUMMARY

Enzymes which hydrolyze dialkyl fluorophosphates appear to be present in all tissues of the human, rat, cat, guinea pig, pigeon, and turtle.

Activation studies show that these enzymes are potentiated by both Mn^{++} and Co^{++}.

Evidence is presented that there are a number of different dialkylfluorophosphatases.

BIBLIOGRAPHY

1. Mazur, A., *J. Biol. Chem.*, **164,** 271 (1946).
2. Augustinsson, K. B., *Biochim. et biophys. acta*, **13,** 303 (1954).
3. Mounter, L. A., Floyd, C. S., and Chanutin, A., *J. Biol. Chem.*, **204,** 221 (1953).
4. Mounter, L. A., *J. Biol. Chem.*, **209,** 813 (1954).
5. Smith, E. L., in Sumner, J. B., and Myrbäck, K., The enzymes, New York, **1,** pt. 2 (1951).
6. Mounter, L. A., and Chanutin, A., *J. Biol. Chem.*, **204,** 837 (1953).

DIA

By L

(*From

It h
fluorop
Since t
it was
demon
some c

Orgs
tone-y
phosph
mediu
and w
a smal
in a co
becaus
rection

Kno
40 mg
side a
(2, 3)
were
hydro
metab
hydro
added
before

The
sicca
final

* Le

XUM

DIALKYLFLUOROPHOSPHATASES OF MICROORGANISMS

By L. A. MOUNTER, ROBERT F. BAXTER,* AND ALFRED CHANUTIN

(From the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia)

(Received for publication, December 23, 1954)

It has been found that enzymes (DFPases) which hydrolyze dialkyl fluorophosphates are present in every tissue of six species studied (1). Since these enzymes may play a rôle in the normal metabolism of tissues, it was decided to study their distribution in microorganisms. These studies demonstrate the presence of DFPases in microorganisms and describe some of their characteristics.

Methods

Organisms were grown for periods varying from 20 to 48 hours in a peptone-yeast extract-glucose medium buffered at pH 6.8 to 7.0 with 0.05 M phosphate buffer. Representative types which grew satisfactorily on this medium were selected for study. Cells were harvested by centrifugation and washed three times with water. The washed cells were suspended in a small volume of water, lyophilized, and stored in a desiccator over P_2O_5 in a cold room (−10°). Lyophilized material was used in all experiments because the results with a given culture were more reproducible and corrections for endogenous metabolism were relatively low.

Known amounts of cells were suspended in 0.025 M $NaHCO_3$, and 20 to 40 mg. of the lyophilized organisms in 0.2 to 0.3 ml. were pipetted into the side arms of the Warburg flasks. The procedures previously described (2, 3) for measuring the hydrolysis of diisopropyl fluorophosphate (DFP) were used. Control experiments were carried out for non-enzymatic hydrolysis ($NaHCO_3$ + DFP) and for the acid production as measured by metabolic CO_2 evolution ($NaHCO_3$ + microorganisms). Results for DFP hydrolysis were corrected for these two blanks. When activators were added to the cell suspensions, they were incubated together for 15 minutes before mixing with the substrate.

Results

The growth of *Proteus vulgaris*, *Pseudomonas aeruginosa*, or *Neisseria sicca* does not appear to be affected when cultured in media adjusted to a final concentration of 10 mM DFP. These organisms are capable of hy-

* Lederle Medical Student Research Fellow, 1954.

XUM

drolyzing DFP, with the liberation of fluoride ion (2, 4). In the inoculated media, almost all of the DFP originally present could be accounted for as free inorganic fluoride; only partial hydrolysis was noted in the uninoculated tubes. Furthermore, the addition of 5×10^{-3} M NaF to the reaction

TABLE I

Hydrolysis of DFP by Microorganisms

Substrate concentration, 10 mM.

Species	Activity, μl. CO_2 per 100 mg. per hr.	Effect of metal ions*		
		Mn^{++}	Co^{++} (10^{-3} M)†	Mg^{++}
Proteus vulgaris (−)	1400	+	−	0
Pseudomonas aeruginosa (−)	1100	+	−	0
Serratia marcescens (−)	900	0	−	0
Salmonella pullorum (−)	650	+	−	+
Pasteurella avicida (−)	470	+	−	−
Escherichia coli (−)	400	+	−	
Shigella alkalescens (−)	270	+	−	0
Aerobacter aerogenes (−)	260	+	−	−
Staphylococcus albus (+)	180	−	−	0
Clostridium sporogenes (+)	170	−	+	0
Corynebacterium xerose (+)	100	+	±	+
Hemophilus pertussis (−)	60			
Bacillus subtilis (+)	50	+	+	+
Neisseria sicca (−)	30	+	+	+
Mycobacterium phlei (+)	<25			
Streptococcus faecalis (folic acid var.) (+)	<25			
" " (+)	<25			
Micrococcus pyogenes (+)	Negligible			
" *citreus* (+)	"			

The symbols in parentheses indicate gram-negative or gram-positive organisms.

* + = activation; − = inhibition; 0 = negligible effect.

† Concentration in the side arm; final concentration after mixing with substrate, 10^{-4} M.

mixture in the Warburg flasks had a negligible effect on the observed DFPase activities.

The DFPase activities of lyophilized microorganisms were determined with and without added Mn^{++}, Co^{++}, or Mg^{++}. DFP was hydrolyzed by all organisms with the exception of two types of micrococci (Table I). These data indicate that the highest activities are possessed by gram-negative organisms. The DFPases in ten of fourteen species were definitely activated by Mn^{++}; this effect was particularly marked in the case of *Salmonella pullorum* and *Shigella alkalescens*. Co^{++} appreciably activated the DFPase of three species, but in most of the organisms this metal ion

exerted a marked inhibitory effect. Mg^{++} had negligible effects in most cases, but inhibited *Aerobacter aerogenes* DFPase.

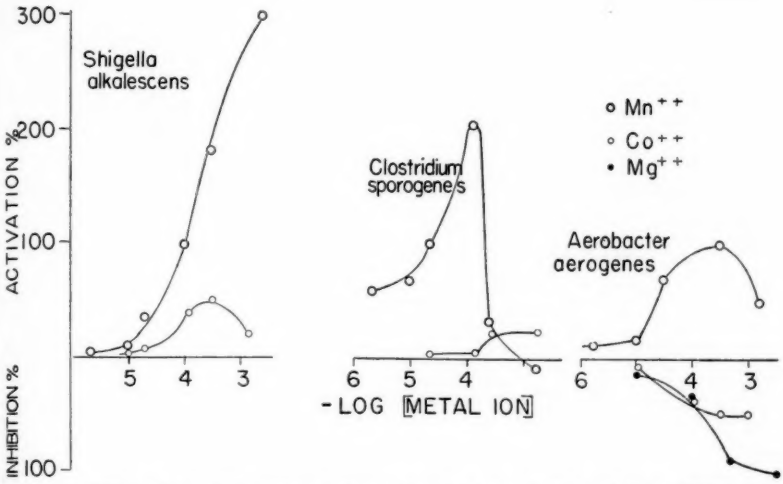


FIG. 1. Effect of varying concentrations of metal ions on the activity of DFPase

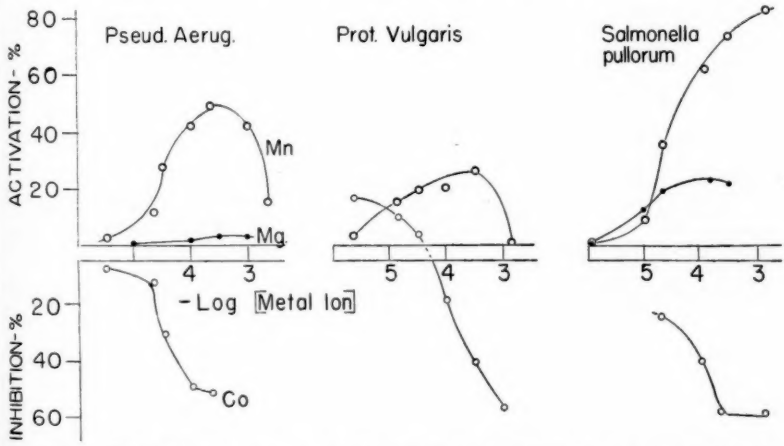


FIG. 2. Effect of varying concentrations of metal ions on the activity of DFPase. Symbols as in Fig. 1.

After this preliminary survey of the DFPase activities, the effects of varying amounts of Mn^{++}, Co^{++}, and Mg^{++} on six selected organisms were studied (Figs. 1 and 2). It can be seen that the degree of activation of the

enzymes by Mn^{++} varies considerably in each case. The DFPases of four microorganisms reached optimal Mn^{++} concentrations between 10^{-3} and

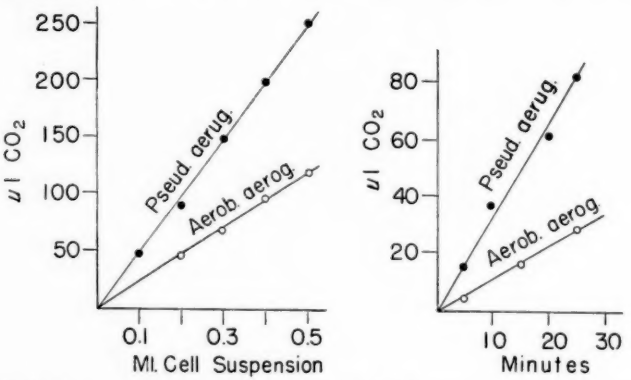


FIG. 3. Influence of cell concentration and time of reaction on the hydrolysis of DFP.

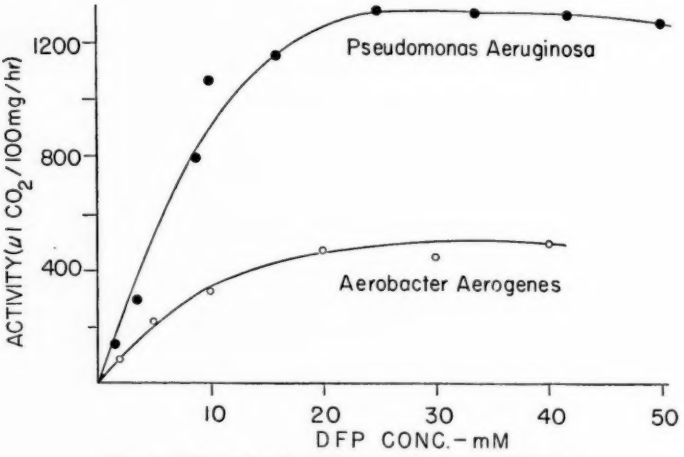


FIG. 4. Effect of substrate concentration on DFPase

10^{-4} M. The results with *Clostridium sporogenes* are particularly striking, since maximal activation and marked inhibition are noted within a small range of Mn^{++} concentration. *Shigella alkalescens* and *Salmonella pullorum* have maximal activities at relatively high concentrations of Mn^{++} (10^{-3} M). Four of the six organisms are inhibited by Co^{++}. Mg^{++} acts as a

powerful inhibitor for the DFPase of *Aerobacter aerogenes* and has slight activation or negligible effects on the remaining organisms.

It has been reported that DFP is hydrolyzed in the presence of microorganisms and that there are species variations in the activation characteristics of the hydrolytic reaction. In order to prove that the observed results are due to enzymes and not to non-specific catalytic effects, a series of kinetic studies was carried out with *Pseudomonas aeruginosa* and *Aerobacter aerogenes*. Linear relationships are observed between CO_2 liberation and both cell concentration and time of reaction (Fig. 3). The effect of substrate concentration on the rate of hydrolysis was determined and, within the limits of experimental accuracy, the points lie on rectangular hyperbolae, which are characteristic of enzyme reactions obeying classical

TABLE II

Inhibition of Bacterial DFPases

	Concentration of inhibitor	Per cent inhibition		
		Staphylococcus albus	*Salmonella pullorum*	*Aerobacter aerogenes*
	M			
Phenylmercuric nitrate	10^{-4}	40	100	55
Cu^{++}	10^{-5}	90	55	60
Sodium arsenite	10^{-3}	45	70	100
Fluoride	5×10^{-4}	10		10
Sequestrene	10^{-3}		85	100
Co^{++}	10^{-3}		80	95
Heat (70° for 5 min.)				70

Michaelis-Menten kinetics (Fig. 4). A number of substances which inhibit the DFPases of tissues (3) have similar effects on the enzymes of the microorganisms (Table II).

DFPase activity in the presence of Mn^{++} and histidine, 2,2′-dipyridyl, or picolinic acid revealed that these cofactors had negligible potentiating effects.

DISCUSSION

It has now been shown that DFPases appear to be universally distributed in vertebrate tissues (1) and in microorganisms. A number of different DFPases are present in microorganisms as judged by their response to metal ion activators and inhibitors. As a result of previous experience with these enzymes, it appears that the characterization of DFPases cannot be made until purified fractions of cell-free extracts are obtained.

According to Goldstein and Goldstein (5), cholinesterases are absent in

most microorganisms. It is noteworthy that bacterial DFPases have the ability to hydrolyze alkyl fluorophosphates, which are powerful inhibitors of cholinesterases. These observations suggest that the two enzymes are not related functionally to each other in tissues.

SUMMARY

Microorganisms are capable of hydrolyzing diisopropyl fluorophosphate, and this reaction may be potentiated or inhibited by Mn^{++}, Co^{++}, or Mg^{++}. Large differences in activity are observed with various types of microorganisms.

Kinetic studies show that DFP is hydrolyzed by enzymes which are similar in many respects to those present in tissues.

The authors wish to express their appreciation to the staff of the Department of Microbiology, and in particular to Dr. Wesley A. Volk, for assistance and helpful advice.

BIBLIOGRAPHY

1. Mounter, L. A., Dien, L. T. H., and Chanutin, A., *J. Biol. Chem.*, **215,** 691 (1955).
2. Mazur, A., *J. Biol. Chem.*, **164,** 271 (1946).
3. Mounter, L. A., Floyd, C. S., and Chanutin, A., *J. Biol. Chem.*, **204,** 221 (1953).
4. Mounter, L. A., and Dien, L. T. H., *Proc. Soc. Exp. Biol. and Med.*, **84,** 40 (1953).
5. Goldstein, D. B., and Goldstein, A., *J. Gen. Microbiol.*, **8,** 8 (1953).

THE COMPLEX NATURE OF DIALKYLFLUOROPHOSPHATASES OF HOG AND RAT LIVER AND KIDNEY

By L. A. MOUNTER

(From the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia)

(Received for publication, December 23, 1954)

Differences in the characteristics of tissue and serum enzymes (DFPases) which hydrolyze diisopropyl fluorophosphate (DFP) and related compounds have been described (1–4). It was shown that DFPases were present in all of the aqueous tissue extracts of six different species, and variations in the degrees of activation and inhibition of these enzymes were noted in the presence of Mn^{++} and Co^{++} (4). The present investigation was undertaken to determine the characteristics of DFPases in the kidney and liver of the hog and rat.

Material and Methods

Enzyme activity was determined by the Warburg technique as described previously (1, 5). The substrates (3 ml.) in the main compartment of the Warburg flask were 0.01 M DFP or di-*n*-butyl fluorophosphate (DBFP).[1] The contents of the side arm are diluted ten times after tipping.

The livers and kidneys of rats were frozen and stored immediately after removal from the animal. Hog tissues were purchased in the frozen state. After thawing, these tissues were minced and homogenized with an equal weight of ice-cold water in the Waring blendor run for 2 minutes at high speed. This homogenate (Fraction H) was further diluted with 4 times the initial weight of the tissues, brought to pH 7.6 with $NaHCO_3$, and centrifuged for 60 minutes at 30,000 $\times$ *g* in a refrigerated Spinco model L centrifuge. The cloudy supernatant solution was removed, and the insoluble residue was twice rehomogenized with water and recentrifuged. The washings were combined with the original supernatant solution (Fraction S) and adjusted to pH 5.8 with acetate buffer (pH 4.0). A precipitate which formed was removed by centrifugation, washed twice with water, and combined with the insoluble residue obtained previously; the first washing was combined with the original supernatant solution. The residue (Fraction I) was suspended in 0.025 M $NaHCO_3$ and dispersed in a

[1] Diisopropyl fluorophosphate was provided by the Medical Division, Army Chemical Corps. Di-*n*-butyl fluorophosphate was prepared by an exchange reaction between sodium fluoride and di-*n*-butyl chlorophosphate (6), which was kindly donated by the Victor Chemical Works, Chicago, Illinois.

Waring blendor run at full speed for about 30 seconds. Kidney and liver residues were dispersed in 2 and 10 times their weight of bicarbonate, respectively. Additional fractions (A and A-1) were obtained by methods described previously (5), in which the DFPase of water-soluble extracts of kidney were purified with ethanol. The enzyme activity of each supernatant solution and fraction was determined with DFP and DBFP.

Results

Activity of DFPases of Aqueous Extracts and Fractions from Kidneys and Livers of Hog and Rat (Table I)—The rates of hydrolysis of DFP and DBFP by enzymes in rat kidney homogenate are about the same. On the other hand, hog kidney DFPase appears to hydrolyze DBFP at a somewhat greater rate than it does DFP. The DBFP:DFP activity ratios of both species remain constant in the presence and absence of Mn^{++}. The insoluble residues of hog and rat kidneys (Fraction I) contain relatively small amounts of DFPase. Mn^{++} potentiates the DFPase activity of all kidney fractions.

The enzymes in the water-soluble fractions (S, A, and A-1) of the livers of both species are activated by Mn^{++}. DFP hydrolysis is potentiated and DBFP hydrolysis is inhibited by the addition of Mn^{++} to the homogenate. The enzymes of Fraction I, in the absence of Mn^{++}, have comparatively slight effect on DFP, but rapidly hydrolyze DBFP; in the presence of Mn^{++} hydrolysis of DBFP is inhibited. These results indicate that there is a marked difference between the DFPases of the livers and kidneys.

Effect of Varying Concentrations of Metal Ions on DFPase Activity—Attempts were made to differentiate the soluble enzymes in Fraction A-1 and insoluble enzymes of Fraction I by determining the effect of varying concentrations of different metal ions on their activities. Since DFP and DBFP were found to be hydrolyzed at maximal rates (Table I) by the enzymes in Fractions A-1 and I, they were used as substrates for the respective fractions. The water-soluble enzymes of the hog and rat kidneys are activated by Mn^{++} and Co^{++}, but are not appreciably affected by Mg^{++} (Fig. 1). The insoluble DFPase of Fraction I, which is present in very small amounts, is slightly activated by Mn^{++} and is little affected by either Co^{++} or Mg^{++}.

The soluble DFPases of the livers are activated in a manner similar to that observed for kidney, but the insoluble DFPases are inhibited by Mn^{++}, Co^{++}, and Mg^{++} (Fig. 2). In view of the marked inhibition of the hydrolysis of DBFP by the liver residues in the presence of Mg^{++}, further studies were undertaken with other metal ions. It was found that DBFP hydrolysis was markedly increased in the presence of Ca^{++}, whereas Fe^{++}, Zn^{++}, and Ni^{++} inhibited the enzyme activity. The results presented in Fig. 3

XUM

TABLE I

DFPase Activity of Kidney and Liver Fractions with DFP and DBFP

Activity in microliters of CO_2 per ml. per 30 minutes. (*a*) = no Mn^{++}; (*b*) = + Mn^{++} 10^{-3} M in the side arm before tipping. H = homogenate; S = supernatant solution; I = insoluble residue.

Fraction	Rat kidney						Hog kidney						Rat liver						Hog liver					
	Activity				DBFP/DFP		Activity				DBFP/DFP		Activity				DBFP/DFP		Activity				DBFP/DFP	
	DFP		DBFP				DFP		DBFP				DFP		DBFP				DFP		DBFP			
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
H	0.5	1.6	0.5	1.7	1.0	1.0	1.8	5.8	2.1	8.4	1.2	1.4	1.0	1.5	4.7	3.6	4.7	2.4	1.6	1.7	3.2	2.2	2.3	1.3
	0.3	1.1	0.3	1.2	1.0	1.1	0.8	1.9	1.2	2.9	1.5	1.5	0.6	1.0	3.5	1.9	6.9	1.9	0.9	1.2	2.9	2.1	3.2	1.8
																			0.4	0.6	1.5	1.5	3.8	2.4
S	0.3	1.6	0.3	1.6	1.0	1.0	1.2	1.8	1.6	3.0	1.3	1.6	0.4	0.9	0.6	1.1	1.5	1.2	1.2	1.4	1.8	1.9	1.5	1.4
							1.0	1.5	1.3	2.1	1.3	1.4	0.6		0.8		1.4							
A	0.2	0.9	0.2	1.1	1.0	1.2	4.0	12.0	5.7	19.0	1.4	1.6	0.2	0.8	0.2	1.0	1.0	1.2	1.7	3.6	1.8	3.9	1.1	1.1
	0.5	2.2	0.5	2.5	1.0	1.1	2.8	12.3	3.5	11.8	1.3	1.0							1.3	1.9	1.6	2.4	1.2	1.3
																			1.2	2.5	1.3	2.7	1.1	1.1
A-1	0.6	3.2	0.7	3.5	1.1	1.1	4.9	11.0	5.6	14.0	1.2	1.3	0.2	2.2	0.2	2.0	1.0	0.9	0.6	1.3	0.8	1.7	1.3	1.3
							4.5	11.4	5.5	13.3	1.2	1.2							0.8	2.9	1.8	3.1	1.2	1.2
I	0.4	1.1	0.4	1.0	1.0	0.9	0.6	0.6	0.8	0.8	1.3	1.3	0.5	0.7	8.4	3.1	16.8	3.4	0.2	0.3	2.1	1.9	11.0	9.0
	0.3	0.8	0.4	0.8	1.2	1.0							0.4		7.2		18.3		0.5	0.9	6.4	5.5	12.8	6.1

show that optimal activation is obtained in the region of 10^{-3} M Ca^{++}. Disodium ethylenediaminetetraacetate (Versene) (10^{-3} M) inhibited the DFPase activity of Fraction I by about 90 per cent, both in the presence

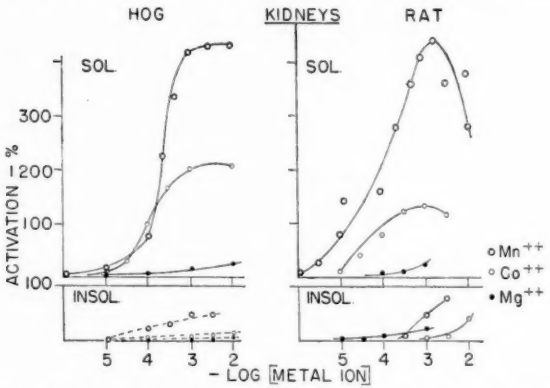


FIG. 1. Effect of varying concentrations of metal ions on the DFPase activity of hog and rat kidney fractions. The concentrations in this and in Figs. 2, 3, and 4 are those in the side arm before tipping.

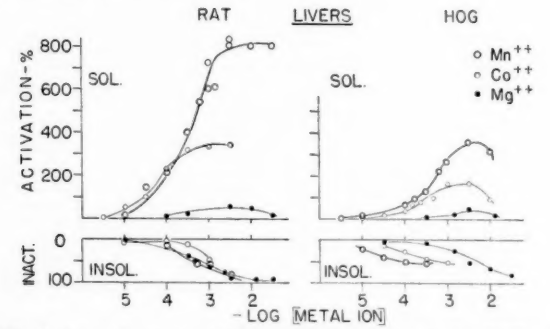


FIG. 2. Effect of varying concentrations of metal ions on the DFPase activity of hog and rat liver fractions.

and absence of Ca^{++}. Citrate, glutamate, and 8-hydroxyquinoline, which form less stable complexes than Versene (7), have negligible effects (Table II).

Effect of Cofactors on Purified Fractions—It has been reported that the DFPase of hog kidney homogenate is activated by a number of amino acids and other organonitrogen compounds in the presence of Mn^{++} and that the degree of activation increases in the course of purification of the

enzyme (5). In the present investigation, it was found that rat kidney homogenate is not significantly affected by histidine, α-picolinic acid, or

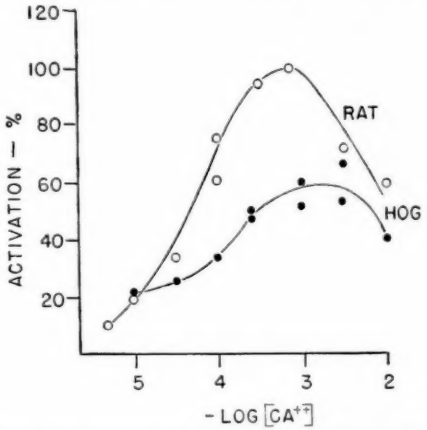


FIG. 3. Effect of varying concentrations of Ca^{++} on the insoluble DFPase of hog and rat liver.

TABLE II

Effect of Calcium-Chelating Agents on Activity of Insoluble Liver DFPases

Chelating agent, 10^{-3} M	Rat		Hog	
	Ca^{++} concentration*	Inhibition	Ca^{++} concentration*	Inhibition
	M	*per cent*	*M*	*per cent*
Disodium ethylenediaminetetraacetate	0	89	0	92
	2.5×10^{-4}	93	3×10^{-4}	43
	8×10^{-4}	93		
Sodium citrate	0	18	0	0
	8×10^{-4}	7	3×10^{-4}	0
Sodium glutamate	0	10	0	0
	8×10^{-4}	0	3×10^{-4}	0
8-Hydroxyquinoline			0	5
			3×10^{-4}	13

* Ca^{++} concentration incubated with enzyme in the side arm for 15 minutes before mixing with substrate.

2,2′-dipyridyl in the presence of Mn^{++}, but the enzyme of the purified fraction (A-1) is readily activated by these cofactors. The potentiating effects by these cofactors (Fig. 4) are similar to those observed with purified hog kidney DFPase (5, 8).

In the presence of Mn^{++}, the DFPase activity of Fractions S, A, and A-1 of rat and hog liver is not influenced by the further addition of cofactors (Table III). On the basis of this evidence, it would appear that the soluble DFPases of the kidney and the liver have different characteristics.

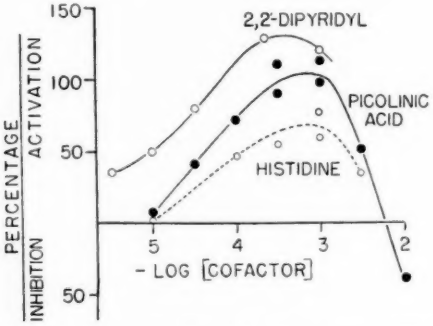


FIG. 4. Effect of varying concentrations of cofactors on rat kidney DFPase (Fraction A-1).

TABLE III

Effect of Activators on Soluble Liver DFPases

The results are in per cent activity.

Addition	Rat liver				Hog liver			
	Homogenate	Supernatant A	Fraction A	Fraction A-1	Homogenate	Supernatant A	Fraction A	Fraction A-1
None	100	100	100	100	100	100	100	100
Mn^{++}	130	150	400	1030	130	130	220	310
Mn^{++} + histidine, 10^{-3} M	130	150		960	120	110	190	310
Mn^{++} + dipyridyl, 10^{-3} M	140	140		1190	120			280

DISCUSSION

The results presented in this paper demonstrate that the liver of the rat and the hog contains at least two different enzymes capable of hydrolyzing dialkyl fluorophosphates. These DFPases differ in solubility, are potentiated by different metal ions, and hydrolyze various alkyl fluorophosphates at different relative rates. On the other hand, only one enzyme, which is water-soluble, has been found in the kidney and may be distinguished from the liver enzymes by activation with amino acids and other organonitrogen compounds.

Any attempt to determine the DFPase content of tissues must be considered as qualitative, since the influence of proteins, metal ions, cofactors, and inhibitors in whole tissue homogenates cannot be properly assessed. It is believed that improved methods of purification may make it possible to demonstrate that DFPases of individual tissues have different properties and characteristics.

SUMMARY

The dialkylfluorophosphatase activities and characteristics of the livers and kidneys of the hog and the rat have been studied.

The water-soluble enzymes of hog and rat livers are activated by Mn^{++} and Co^{++} and hydrolyze diisopropyl fluorophosphate and di-*n*-butyl fluorophosphate at about the same rates. The insoluble enzymes of these livers hydrolyze DBFP at a considerably greater rate than they do DFP and are activated by Ca^{++} and inhibited by Mn^{++}, Co^{++}, and Mg^{++}. Amino acids and other organonitrogen compounds do not potentiate the activities of liver enzymes.

A single water-soluble DFPase appears to be present in the kidneys. Rat kidney DFPase is activated by Mn^{++} and by Mn^{++} plus organonitrogen compounds.

The author wishes to express his gratitude to Mrs. Lien Tien H. Dien for valuable technical assistance.

BIBLIOGRAPHY

1. Mazur, A., *J. Biol. Chem.*, **164,** 271 (1946).
2. Augustinsson, K. B., *Biochim. et biophys. acta*, **13,** 303 (1954).
3. Mounter, L. A., *J. Biol. Chem.*, **209,** 813 (1954).
4. Mounter, L. A., Dien, L. T. H., and Chanutin, A., *J. Biol. Chem.*, **215,** 691 (1955).
5. Mounter, L. A., Floyd, C. S., and Chanutin, A., *J. Biol. Chem.*, **204,** 221 (1953).
6. Kosolapoff, G. M., The organophosphorus compounds, New York (1950).
7. Martell, A. E., and Calvin, M., Chemistry of the metal chelate compounds, New York (1952).
8. Mounter, L. A., and Chanutin, A., *J. Biol. Chem.*, **210,** 219 (1954).

METABOLISM OF 11-DEOXYCORTISOL IN VITRO*

By ENRICO FORCHIELLI, HARRIS ROSENKRANTZ, AND RALPH I. DORFMAN

(From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts)

(Received for publication, November 12, 1954)

In this study 11-deoxycortisol (17α,21-dihydroxy-Δ^4-pregnene-3,20-dione) was incubated with a partially purified preparation derived from rat liver with the formation of ring A reduced steroids of the allopregnane (5α) type. Similar reductions of ring A have been previously observed by Schneider (1) who incubated deoxycorticosterone with rat liver slices and by Caspi *et al.* who perfused cortisone (2) and cortisol[1] through rat livers. Isselbacher and Tomkins (3, 4) incubated cortisone with purified rat liver preparation and demonstrated reduction to the pregnane (5β) series of steroids. In addition to the conversion of 11-deoxycortisol to allopregnane steroids, the formation of 3α-hydroxy and 3β-hydroxy compounds, a 20β-hydroxy compound, and two C_{19} steroids is demonstrated in this report.

Methods

Male and female albino rats weighing approximately 300 gm. were sacrificed and bled. The livers were removed within 1 minute, placed on ice until the collection was complete, and homogenized for 30 seconds in a Waring blendor at 5° in a medium (1.0 ml. of medium per gm. of liver) containing 0.1 M phosphate buffer at pH 7.35, 0.02 M glutamate, and 0.01 M nicotinamide. The homogenate was strained through gauze and centrifuged at 6000 $\times$ g for 30 minutes and the residue was separated from the supernatant fluid.

20 ml. of the supernatant fluid were added to 125 ml. Erlenmeyer flasks containing 15 mg. of steroid dissolved in 0.9 ml. of propylene glycol. Neutralized diphosphopyridine nucleotide (DPN) was added to each flask to a final concentration of 0.002 M immediately prior to incubation. The volume of the supernatant fluid used per flask represented an initial tissue-steroid ratio of 1000:1. The incubations were carried out in open flasks in a Warburg apparatus for 2 hours at 38°.

The incubation mixture was diluted with 10 volumes of acetone to pre-

* This work was supported (in part) by contract No. DA-49-007-MD-184, Medical Research and Development Board, Office of the Surgeon General, Department of the Army, and by a grant-in-aid, No. 36, Committee on Research of the Council on Pharmacy and Chemistry of the American Medical Association.

[1] Caspi, E., personal communication.

XUM

cipitate the proteins and stirred at 5° overnight. The acetone-aqueous extract was separated from the precipitated protein by filtration on a Büchner funnel and washed with hot acetone. The combined filtrates were concentrated *in vacuo* and the resulting aqueous concentrate was made up to 70 per cent methanol and partitioned against equal volumes of ligroin three times. The pooled ligroin fractions were washed with 0.5 volume of 70 per cent methanol and the aqueous-methanol extracts were combined and concentrated *in vacuo*. This second aqueous concentrate was extracted three times with equal volumes of chloroform, the chloroform extracts being combined and washed twice with 0.05 volume of distilled water, dried over sodium sulfate overnight, decanted, concentrated *in vacuo*, and finally dried in a stream of nitrogen at 35°.

An initial crude fractionation of extracts was carried out by adsorption on a silica gel column (Davidson silica gel, 200 mesh) and elution in succession with benzene, benzene-ethyl acetate mixtures, and finally ethyl acetate. Paper partition chromatography with the toluene-propylene glycol (5) and the ligroin-propylene glycol systems (6) was employed to screen the silica gel fractions. This technique provided a guide for pooling the silica gel fractions containing similar components, as indicated by mobility and color reactions on the paper. The pooled fractions so obtained were further resolved by preparative paper chromatography on paper strips 15 cm. in width in the above systems. The paper chromatograms were developed from 10 to 72 hours with a run off fraction being collected. The strips were dried overnight in a hood at room temperature. 2 mm. strips were cut along the length of the chromatograms and exposed to dinitrophenylhydrazone reagent (DNPH) (7), triphenyltetrazolium chloride (TPTZ) (8), Zimmermann reagent, and the ultraviolet lamp (9). This procedure permitted positioning of the reactive zones and indicated the areas to be cut from the paper strip for elution with methanol-methylene dichloride (1:1).

The isolated products were examined in the solid state and in carbon disulfide solution in the infra-red on a 12C Perkin-Elmer spectrophotometer. Sulfuric acid chromogen determinations were made with a Cary 11MS spectrophotometer. Melting points were obtained on a Kofler block and are reported uncorrected.

EXPERIMENTAL

Incubation of 885 mg. of 11-deoxycortisol was performed with 1450 ml. of supernatant fluid obtained by homogenizing 1200 gm. of rat livers with 1200 ml. of buffer media. After extraction, defatting, and drying, 1.98 gm. of crude extract were obtained. The extract was adsorbed on a 120 gm. column of silica gel and eluted with 100 ml. volumes as indicated in Table I.

Aliquots of the above silica gel eluates were applied to 1 cm. strips of paper and developed in the toluene-propylene glycol system and the results

TABLE I

Initial Fractionation with Silica Gel Column

Fraction Nos.	Solvent systems	Weight
		mg.
1– 5	Benzene (500 ml.)	105.5
6– 9	Benzene-ethyl acetate, 19:1	95.2
10–13	" " 14:1	40.8
14–17	" " 9:1	5.3
18–21	" " 4:1	12.0
22–23	" " 3:1	6.0
24–44	" " 2:1	392.6
45–51	" " 1:1	50.5
52–57	Ethyl acetate	211.6
58–59	" acetate-methanol, 99:1	28.1
60–65	" " 99:2	44.3
66–75	" " 97:3	214.0
76	Methanol	138.0

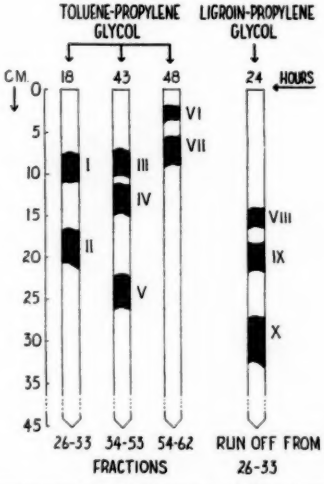


FIG. 1. Paper chromatography of aliquots of fractions from silica gel column chromatography.

are illustrated in Fig. 1 and the various detection reactions are listed in Table II. The presence of steroid material was indicated in Fractions 26 to 62 and the total crude weight of the combined corresponding fractions

from the silica gel column accounted for approximately 30 per cent of the weight initially adsorbed on the column.

11-Deoxycortisol and 17α,21-Dihydroxyallopregnane-3,20-dione

Fractions 26 to 33 were pooled (127 mg.) and chromatographed in the toluene-propylene glycol system for 18 hours. Two components were noted: the unchanged starting material, which was isolated and identified by spotting reactions on paper, mobility, and infra-red analysis (24 mg. being recovered in this group of fractions), and 17α,21-dihydroxyallopregnane-3,20-dione. This latter compound (m.p. 201–207°) was identified on paper (reduced TPTZ, yellow DNPH reaction, not visible under the ultraviolet lamp) and by comparison of its infra-red spectrum with that of

TABLE II

Reactions on Paper of Various Zones Illustrated in Fig. 1

Zone No.	TPTZ	DNPH	Zimmermann	Ultraviolet lamp
I	+	Orange		+
II	+	Yellow		−
III	+			−
IV	+			−
V	+	Orange		+
VI	+			−
VII	−	Orange		+
VIII	−		+	−
IX	−	Orange	+	+
X	−		+	−

an authentic sample of 17α,21-dihydroxyallopregnane-3,20-dione, m.p. 203–208° (Fig. 2). The total yield of this compound was 50 mg., 8 per cent of the recovered material.

3β,17α,21-Trihydroxyallopregnan-20-one and 3α,17α,21-Trihydroxyallopregnan-20-one

Fractions 34 to 53 were combined (321 mg.) and chromatographed for 72 hours in the toluene-propylene glycol system. Two TPTZ-reacting zones were found. The material from the slower moving zone was chromatographed on a 9 gm. silica gel column and 65 mg. of crystalline material were obtained from the combined eluates with benzene-ethyl acetate (2:1). 39 mg. of this material were further separated with digitonin.

16 mg. of the digitonin-precipitable material were crystallized from absolute ethanol three times, yielding 5 mg. of colorless needles melting at 210–213°. On paper this material gave a positive reaction with TPTZ,

no DNPH reaction, and could not be visualized under the ultraviolet lamp. The acetate was prepared in the usual way and the infra-red spectra of the free compound and the acetate derivative (m.p. 198–200°) proved to be identical to those of 3β,17α,21-trihydroxyallopregnan-20-one (m.p. 214–216°) and its diacetate (m.p. 198–201°), respectively (Fig. 3).

The material not precipitable by digitonin was crystallized from absolute ethanol, yielding 8 mg. of crystals, m.p. 203–213°. The TPTZ reaction on paper was positive, but the material could not be visualized under the ultraviolet lamp and the DNPH reaction was negative. The infra-red spectrum of this compound was compared with the spectra of three tetrahydro com-

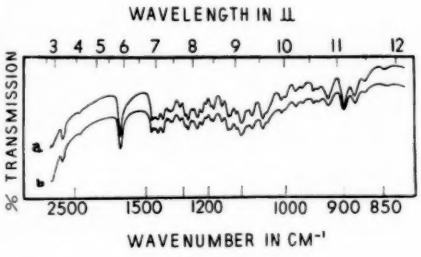


FIG. 2

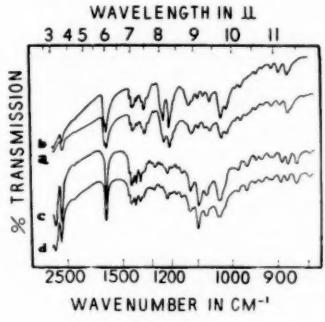


FIG. 3

FIG. 2. Comparison of the infra-red spectra of 17α,21-dihydroxyallopregnane-3,20-dione, (*a*) isolated and (*b*) authentic samples.

FIG. 3. Comparison of the infra-red spectra of 3β,17α,21-trihydroxyallopregnan-20-one, (*a*) prepared 3,21-diacetate, (*b*) authentic 3,21-diacetate, (*c*) isolated and (*d*) authentic samples.

pounds available as reference substances, 3β,17α,21-trihydroxyallopregnan-20-one, 3α,17α,21-trihydroxypregnan-20-one, and 3β,17α,21-trihydroxypregnan-20-one. Significant spectral differences indicated that the compound was not identical with the available tetrahydro compounds. However, structural interpretations based on the infra-red spectra of the isolated steroid and its diacetate were possible (Fig. 4). The presence of a non-conjugated hexacyclic carbonyl group was indicated by a band near 1709 cm.$^{-1}$, while an absorption band near 3392 cm.$^{-1}$ was assigned to hydroxyl vibrations. An intense band near 1009 cm.$^{-1}$ (10) in the spectrum of the free compound and a complex acetate band in the 1250 cm.$^{-1}$ region (11), in addition to an absorption near 1018 cm.$^{-1}$ (12) in the spectrum of the acetate derivative, were strong evidence for the presence of a C_3-α-hydroxy group cis to a C_5-hydrogen. The absence of absorption near 1666 cm.$^{-1}$ indicated a reduced structure, since unsaturated groupings give rise

to intense absorption bands near this wave-length. Oxidative degradation of the α-ketol side chain with sodium bismuthate according to the method of Brooks and Norymberski (13) yielded the 17-ketosteroid androsterone as the end-product, which was identified by infra-red analysis and mobility on paper. Although a reference substance was not available for unequivocal identification, all the data indicated that the compound was $3\alpha,17\alpha$,-21-trihydroxyallopregnan-20-one. The conversion of 11-deoxycortisol to the 3β-tetrahydro compound was of the order of 4 per cent and to that of the 3α-tetrahydro compound 6 per cent. In the run off fraction 120 mg. of starting material were recovered.

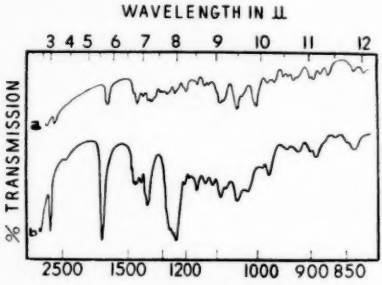


FIG. 4

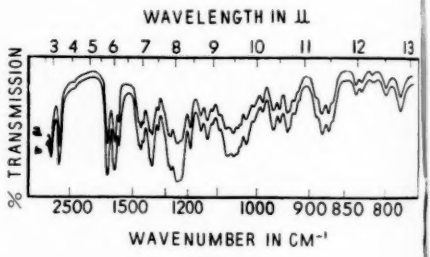


FIG. 5

FIG. 4. Infra-red spectra of (*a*) $3\alpha,17\alpha,21$-trihydroxyallopregnan-20-one and (*b*) its 3,21-diacetate.

FIG. 5. The infra-red spectra of (*a*) $17\alpha,20\beta,21$-trihydroxy-Δ^4-pregnen-3-one-20,21-diacetate and (*b*) an authentic sample of the 20,21-diacetate.

17α,20β,21-Trihydroxy-Δ⁴-pregnen-3-one

Because of the high polarity of the combined components comprising Fractions 54 to 62 (185 mg.) this material was chromatographed for 10 hours in the chloroform-formamide system (14). Two areas were detected. The faster moving material gave a dark band under the ultraviolet lamp, a positive DNPH reaction, and was negative to the TPTZ reagent. The slower moving component gave a positive TPTZ reaction, a faint yellow DNPH reaction, and was not visible under the ultraviolet lamp. This material has not been as yet identified. The faster moving component was eluted from paper, acetylated, and crystallized from absolute ethanol, yielding 6 mg. of colorless needles (m.p. 189–191°). The infra-red spectrum of the acetate was identical to that of an authentic sample of $17\alpha,20\beta,21$-trihydroxy-Δ^4-pregnen-3-one (m.p. 190–193°) (Fig. 5). The total yield was approximately 4 per cent.

Androsterone and Δ^4-Androstene-3,17-dione

The run off collected when Fractions 26 to 33 were chromatographed for 18 hours in the toluene-propylene glycol system was rechromatographed in ligroin-propylene glycol system. Three zones having a positive Zimmermann reaction were found. The fastest moving zone has not yet been identified. The intermediate zone, in addition to giving a positive Zimmermann reaction, was visible under the ultraviolet lamp and gave an orange DNPH reaction. After elution from paper, its infra-red and sulfuric acid spectra and mobility rate on paper were found to be identical with those of an authentic sample of Δ^4-androstene-3,17-dione. The slowest moving Zimmermann-positive zone, after elution from paper, had an infra-red spectrum, a sulfuric acid spectrum, and a mobility rate on paper identical with those of an authentic sample of androsterone. The yield of each of these compounds was about 0.1 per cent of recovered material.

DISCUSSION

This study has demonstrated that rat liver supernatant fluid prepared by centrifugation at 6000 $\times$ g contains five different reducing enzyme systems. The reactions that have been demonstrated in this study for 11-deoxycortisol are listed in Table III and are compared to those previously reported in the literature for various corticoids. Of particular interest is the fact that in this study, as well as in the reports of Caspi *et al.* and Schneider, the reduction in ring A led exclusively to the allopregnane (5α) form. When Tomkins and Isselbacher centrifuged a rat liver homogenate at 78,000 $\times$ g, the supernatant fluid contained an enzyme system which reduced ring A of cortisone exclusively to the pregnane (5β) form. On the basis of these facts it is suggested that the crude homogenate contains two separate enzyme systems, one which catalyzes the 5α reduction and the other which catalyzes the 5β reduction. Possibly, the two enzymes can be separated by further centrifugation. If two separate enzyme systems (the 5α and 5β reducing systems) do actually exist in rat liver, which seems likely, why does perfusion of the intact liver, the use of slices, or even the use of the homogenate supernatant fluid (6000 $\times$ g) lead exclusively to the 5α stereoisomeric form? This question cannot be answered at this time. The presence of an inhibitor of the 5β reducing enzyme system which is separated into the residue fraction by a force of 78,000 $\times$ g could explain the facts.

In this work, incubation of 11-deoxycortisol with the supernatant fluid from rat liver homogenate yielded the dihydro derivative as the major metabolite. In contrast, Caspi *et al.* (2), in the perfusion of cortisone through the intact rat liver, isolated no dihydro derivative; Schneider (1) was able to isolate only minor amounts of the dihydro derivative of deoxy-

corticosterone with liver slices. It may be interpreted that, with increasing disruption of cellular organization, the availability of a cofactor (or cofac-

TABLE III

*Reactions of Corticoids Demonstrated by Methods in Vitro with Rat Liver Preparations**

Reactions	Substrates				
	Deoxycorticosterone	Cortisone	Cortisol	17α,21-Dihydroxypregnane-3,20-dione	11-Deoxycortisol
3-C=O → 3α-C—OH	Schneider (1)†	Caspi *et al.* (2), Tomkins and Isselbacher (4)		Ungar and Dorfman (15)	This report
3-C=O → 3β-C—OH	Schneider (1)	Caspi (2)	Caspi‡		This report
Δ^4 → saturated allopregnane (5α)	Schneider (1)	" (2)	" ‡		This report
Δ^4 → saturated pregnane (5β)		Tomkins and Isselbacher (4)			
20-C=O → 20β-C—OH	Schneider (1)	Caspi (2)	Caspi‡		This report
11-C=O → 11β-C—OH		" (2)			
CH_2OH | C=O → 17-keto-steroid | C···OH		" (2)	Caspi‡		This report

* The type of liver preparation employed in each investigation is as follows: Schneider (1), slice; Caspi *et al.* (2), perfused organ; Tomkins and Isselbacher (4), purified supernatant fluid; Ungar and Dorfman (15), purified supernatant fluid; present study, supernatant.

† Bibliographic reference No.

‡ Personal communication. Cortisol was perfused through an intact liver.

tors) may be limiting in the reaction involving the reduction of the 3-ketone group to the 3α- or 3β-hydroxy group.

Attack at the C_{20}-carbonyl appeared to proceed exclusively to the 20β-hydroxy stereoisomer which is supported by the work of Schneider (1), Caspi *et al.* (2), and Ungar.[2] The latter worker obtained 3α,20β-dihy-

[2] Ungar, F., personal communication.

droxypregnane from the incubation of pregnanolone with rat liver homogenate.

Although the conversion of the C_{21} steroid 11-deoxycortisol to the C_{19} steroids androsterone and Δ^4-androstene-3,17-dione was of a very small order, this is the first reported instance of oxidative degradation of the α-ketol side chain by means of a liver homogenate. The significance of this lies in the fact that the enzyme systems responsible for the formation of

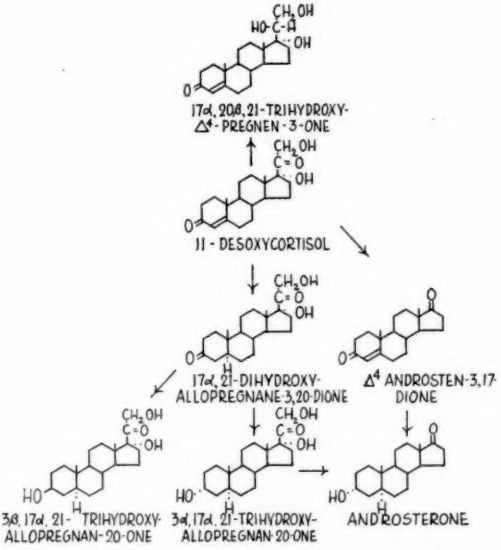


FIG. 6. Transformation scheme for the metabolism of 11-deoxycortisol by rat liver tissue.

C_{19} compounds from C_{21} compounds are accessible in liver tissue and the possibility exists for studying the mechanism of this reaction.

It should be pointed out that only ketonic steroids were visualized and isolated and no attempt was made to isolate and identify any non-ketonic steroids that may have been formed.

In conclusion a tentative scheme is presented (Fig. 6) for the stepwise metabolism of 11-deoxycortisol by rat liver tissue *in vitro*.

SUMMARY

1. When 11-deoxycortisol was incubated with a supernatant fluid from rat liver homogenate, six metabolites were isolated and identified by infrared analysis: 3β,17α,21-trihydroxyallopregnan-20-one, 3α,17α,21-trihydroxyallopregnan-20-one, 17α,21-dihydroxyallopregnane-3,20-dione, 17α,-

20β,21-trihydroxy-Δ^4-pregnen-3-one, Δ^4-androstene-3,17-dione, and 3α-hydroxyandrostan-17-one.

2. In addition to enzymes capable of reducing the ring A double bond, the C_3 ketone, and the C_{20} ketone, the presence of enzymes capable of oxidative removal of the α-ketol side chain has been demonstrated.

3. With the tissue preparation employed in this work, all the reduced metabolites of ring A isolated and identified were exclusively of the allopregnane (5α) type.

We are indebted to Syntex, S. A., and the Chemical Specialties Company, Inc., for generous samples of 3β,17α,21-trihydroxyallopregnan-20-one-3,21-diacetate and 17α,21-dihydroxyallopregnane-3,20-dione and to Ciba Pharmaceutical Products, Inc., for 17α,20β,21-trihydroxy-Δ^4-pregnen-3-one-20,21-diacetate. We are grateful to Dr. Mika Hayano for her aid in setting up the initial incubation experiments and to Mr. Paul Skogstrom for the infra-red analyses.

BIBLIOGRAPHY

1. Schneider, J. J., *J. Biol. Chem.*, **199,** 235 (1952).
2. Caspi, E. Y., Levy, H., and Hechter, O. M., *Arch. Biochem. and Biophys.*, **45,** 169 (1953).
3. Isselbacher, K. J., and Tomkins, G., *Federation Proc.*, **13,** 236 (1954).
4. Tomkins, G., and Isselbacher, K. J., *J. Am. Chem. Soc.*, **76,** 3100 (1954).
5. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.*, **193,** 749 (1951).
6. Savard, K., *J. Biol. Chem.*, **202,** 457 (1953).
7. Zaffaroni, A., Burton, R. B., and Keutmann, E. H., *Federation Proc.*, **9,** 250 (1950).
8. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, **188,** 763 (1951).
9. Tennet, D. H., Whitla, J. B., and Florey, K., *Anal. Chem.*, **23,** 1748 (1951).
10. Rosenkrantz, H., and Zablow, L., *J. Am. Chem. Soc.*, **75,** 903 (1953).
11. Jones, R. N., Humphries, P., Hurling, F., and Dobriner, K., *J. Am. Chem. Soc.*, **73,** 3215 (1951).
12. Rosenkrantz, H., and Skogstrom, P., *J. Am. Chem. Soc.*, in press.
13. Brooks, C. J. W., and Norymberski, J. K., *Chem. and Ind.*, 804 (1952).
14. Zaffaroni, A., *Recent Progress Hormone Res.*, **8,** 51 (1953).
15. Ungar, F., and Dorfman, R. I., *J. Am. Chem. Soc.*, **76,** 1197 (1954).

GASTROINTESTINAL DIGESTION OF STARCH

I. THE ACTION OF OLIGO-1,6-GLUCOSIDASE ON BRANCHED SACCHARIDES

By JOSEPH LARNER and C. M. McNICKLE*

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois)

(Received for publication, December 3, 1954)

Digestion of starch in mammals occurs chiefly in the mouth and small intestine through the catalytic activity of α-amylase and maltase (α-glucosidase). Extensive α-amylolysis of amylose and amylopectin results in the formation of maltose, glucose, and a mixture of branched oligosaccharides (α-amylase dextrins) (1). The latter compounds, whose structure is currently being investigated by several groups (2, 3), contain the α-1,6 linkages of the amylopectin fraction intact, since these linkages are not subject to hydrolysis by α-amylase (4). Meyer and Gonon (4) have recently suggested that, if hydrolysis of amylopectin by α-amylase is carried out to completion, isomaltose is the sole branched product.

Glucose is subsequently absorbed through the intestinal wall. Maltose is hydrolyzed to glucose by intestinal maltase and absorbed as glucose (5). Any maltose which escapes hydrolysis in the intestine is subsequently hydrolyzed by maltase present in the blood (5). We have recently shown (6) that intestinal mucosa contains a new enzyme termed oligo-1,6-glucosidase, which specifically hydrolyzes the α-1,6 linkages of isomaltose, "panose," and α-amylase dextrins.[1] The three enzymes, α-amylase, oligo-1,6-glucosidase, and maltase, catalyze the essentially complete digestion of starch in the gastrointestinal tract.

Oligo-1,6-glucosidase differs in its action from amylo-1,6-glucosidase and R enzyme. It is the purpose of the present report to describe the activity and properties of this new enzyme.

* This work was supported in part by a grant from the Graduate College of the University of Illinois, Urbana.

[1] Since the previous work was published (6), a report of similar enzymatic activity has come to our attention (7). An enzyme from bovine intestinal juice and intestinal mucosa, tentatively called α-limitdextrinase, was shown by increase in reducing power alone to hydrolyze the α-amylase dextrins. No separation from maltase activity was achieved. For further details, see "Discussion."

Methods and Materials

Enzymes and Coenzymes—Extracts were prepared from hog small intestine obtained as soon as possible from the slaughter-house.[2] All steps were carried out in the cold room at 3°. The intestines were opened, washed with cold distilled H_2O, and the mucosa removed by scraping with a knife. The scrapings (usually 50 gm.) were thoroughly ground in a mortar with an equal weight of washed sand. The mass was extracted by continued grinding with 5 volumes of cold distilled water and clarified by centrifugation at the highest speed of the Servall centrifuge, model SS-1, for 30 minutes. The opalescent reddish fluid was filtered through Whatman No. 1 paper and dialyzed for 4 to 6 hours against 0.85 per cent NaCl. It was stored at 3° with a small test-tube containing toluene suspended over the surface. When prepared and stored in this manner, the enzyme usually retained full activity for 3 weeks.

Hexokinase from brewers' yeast was prepared by an unpublished method supplied by C. R. Park of Vanderbilt University. Concentrated stock solutions were diluted with 1 per cent amorphous insulin, frozen in small aliquots, and used as needed.[3] *Zwischenferment* was purified from yeast by the method of Kornberg (8). Amylo-1,6-glucosidase was prepared from pooled supernatant fluids remaining after the crystallization of muscle phosphorylase, as previously described (9). Adenosine triphosphate was purchased from the Pabst Laboratories, and triphosphopyridine nucleotide (TPN) from the Sigma Chemical Company or prepared by the method of Kornberg and Horecker (10).

Substrates—α-Amylase dextrins were prepared by extensive action of salivary amylase on waxy maize amylopectin.[3] Two digestions were carried out with undiluted saliva. 1 ml. of filtered saliva was incubated with 186 mg. of amylopectin at 30° for 25.5 hours, at which time digestion had proceeded to the extent of 44.0 per cent in terms of glucose equivalents. It was treated a second time with 0.5 ml. of filtered saliva for 16 hours at 30°, during which time digestion proceeded an additional 5.7 per cent. Enzymatic activity was stopped by heating at 100°. Glucose and maltose were removed by four fermentations with bakers' yeast. The absence of these two sugars was checked by paper chromatography. The average

[2] Older hogs, weighing 100 pounds or more, seem to be a better source of enzyme than younger animals.

[3] We gratefully acknowledge the gift of the following materials: amorphous insulin from Dr. O. K. Behrens, Eli Lilly and Company; waxy maize amylopectin from Dr. T. J. Schoch, Corn Products Refining Company; isomaltose from Dr. Allene Jeanes, Northern Utilization Research Branch; panose from Dr. S. C. Pan, The Squibb Institute for Medical Research; and clinical dextran from Dr. E. B. Hodge, Commercial Solvents Corporation.

degree[4] of polymerization of the branched dextrins was 6.5. The mixture of "branched" oligosaccharides which contained isomaltose and at least three higher saccharides was stored in the frozen state and used as needed. Muscle phosphorylase limit dextrin was prepared as previously described (9).

Analytical—Paper chromatography was carried out by the multiple ascent method described by French and coworkers (11) and Jeanes and coworkers (12). The chromatograms were sprayed with alkaline 3,5-dinitrosalicylate according to Jeanes and coworkers (12), or with ammoniacal $AgNO_3$ according to the method of Hough (13). Reducing sugars were determined by the Nelson method (14) with the alkaline Reagent 60 of Shaffer and Somogyi (15) with the longer boiling time of 30 minutes. Deproteinization was accomplished by the $Ba(OH)_2$-$ZnSO_4$ method of Somogyi (16) with the modifications suggested by Hers and Kusaka (17).

Protein was determined by the biuret method of Robinson and Hogden (18). In some cases protein was determined nephelometrically after precipitation by trichloroacetic acid essentially by the method suggested by Burton and Stadtman (19). Crystalline bovine serum albumin was used as the standard. Phosphate was determined by the method of Fiske and Subbarow (20).

Hydrolysis of Isomaltose, Panose, and α-Amylase Dextrins

Incubation of intestinal extracts with isomaltose, panose, or the α-amylase dextrins leads to a rapid increase in reducing power. Typical experiments are shown in Fig. 1. Substrates were incubated with enzyme in the absence of buffer at 30°. Aliquots were removed at zero time and at time intervals, deproteinized by the $Ba(OH)_2$-$ZnSO_4$ method, and the reducing power was determined. Essentially complete hydrolysis of isomaltose (Curve 1) (98.5 per cent) and the α-amylase dextrins (Curve 3) (103 per cent) had occurred in 210 and 30 minutes, respectively. Panose (Curve 2) was hydrolyzed 87.5 per cent in 180 minutes. All hydrolyses are recorded in terms of glucose equivalents. Since these experiments were designed to produce essentially complete hydrolysis, the amounts of enzyme and substrate added vary, and the rates of hydrolysis are therefore not comparable. Seiji (7), using α-amylase dextrins as substrate, records hydrolyses in the range of 12.4 to 58.5 per cent in terms of glucose equivalents.

In order to rule out phosphorolytic reactions, intestinal extracts were dialyzed to remove inorganic phosphate. Before dialysis, analysis for in-

[4] Determined by increase in reducing power after acid hydrolysis. Meyer and Gonon (4) report that 50 per cent hydrolysis of amylopectin by crystalline pancreatic α-amylase liberates branched dextrins of a degree of polymerization of 6.0.

organic phosphate revealed 125 to 150 γ per ml. After 4 to 6 hours of dialysis against 0.85 per cent NaCl, these levels had decreased to 4.7 and 9.8 γ per ml. Enzymatic activity was not decreased by dialysis. In addition, deproteinization was routinely carried out by the $Ba(OH)_2$-$ZnSO_4$ method of Somogyi, which removes phosphorylated sugars.

Bacterial Contamination—That intact bacteria are not directly responsible for enzymatic activity is shown by the fact that enzymatic activity was retained after filtration through a bacteriological filter (sintered glass). For example, incubation of 1.0 ml. of intestinal extract (1.97 mg. of protein)

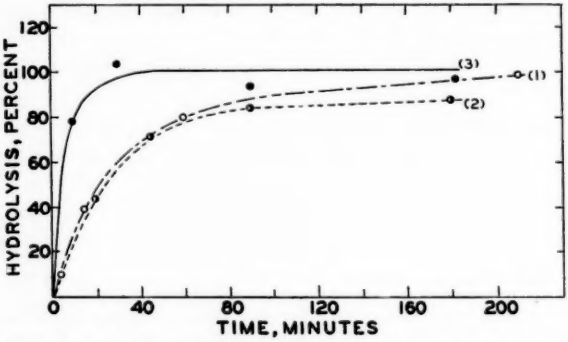


FIG. 1. Hydrolysis of branched saccharides by oligo-1,6-glucosidase. Curve 1, isomaltose. The isomaltose reaction mixture contained 2 mg. of isomaltose, 58 units of oligo-1,6-glucosidase; volume 2.2 ml. Curve 2, panose. The panose reaction mixture contained 15 mg. of panose, 177 units of oligo-1,6-glucosidase; volume 6.2 ml. Curve 3, α-amylase dextrins. The α-amylase dextrin reaction mixture contained 0.199 mg. of α-amylase dextrins, 216 units of oligo-1,6-glucosidase; volume 6.2 ml. For the definition of a unit, see the text.

with 4.3 mg. of panose at 30° for 210 minutes resulted in 37.5 per cent hydrolysis before filtration, and 16.2 per cent after filtration through a bacteriological filter. The loss in activity is explained by the fact that the extract had been frozen and thawed between the two experiments, a procedure known to reduce oligo-1,6-glucosidase activity in crude extracts. Also, after centrifugation at the highest speed of Servall model SS-1 for 30 minutes, extracts had between 10^5 and 10^6 organisms per ml., which decreased to 10^3 organisms per ml. after storage for 18 hours in the presence of toluene.[5] Assuming 10^{12} organisms per gm., 10^6 organisms per ml. would amount to less than 0.01 per cent of the protein present in the extracts (usually about 10 mg. of protein per ml.). However, the possi-

[5] We wish to thank J. R. Stamer and I. C. Gunsalus of the Department of Bacteriology for carrying out these counts.

bility that enzymatic activity is of bacterial origin is still open to question. This possibility seems less likely in view of the fact that extracts made from hogs which had received antibiotics in the ration sufficient to elicit a growth response had enzyme activities similar to controls which had received no antibiotics.[6] There was also no significant difference in the activity of extracts prepared by grinding with sand in the presence of toluene. However, to arrive at a more definite conclusion, this problem is currently being investigated with germ-free animals.

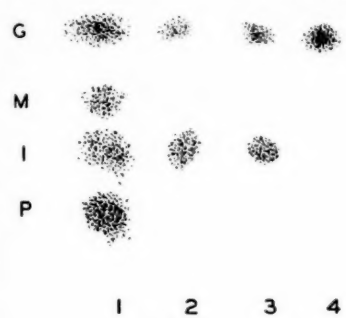


FIG. 2

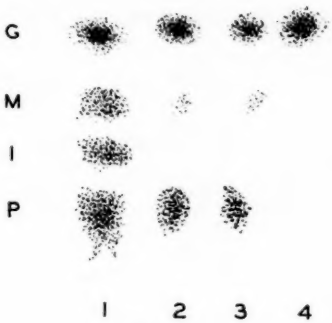


FIG. 3

FIG. 2. Hydrolysis of isomaltose by oligo-1,6-glucosidase. Amount of stippling roughly proportional to intensity of spot on original chromatogram. G, glucose; M, maltose; I, isomaltose; P, panose; Column 1, controls; Column 2, 34.2 per cent hydrolysis; Column 3, 54.5 per cent hydrolysis; Column 4, 95.0 per cent hydrolysis.

FIG. 3. Hydrolysis of panose by oligo-1,6-glucosidase. Letter designations are the same as in Fig. 2. Column 1, controls; Column 2, 49.5 per cent hydrolysis; Column 3, 73.8 per cent hydrolysis; Column 4, 97.5 per cent hydrolysis.

Paper Chromatography—Product formation was investigated by three methods; fermentability by bakers' yeast, paper chromatography, and coupling with a specific microenzymatic test system.

The product formed after the hydrolysis of isomaltose was completely fermentable by bakers' yeast. Paper chromatography (Fig. 2) revealed that the only product which appeared either at intermediate stages or after essentially complete hydrolysis of isomaltose was free glucose.

At intermediate stages of the hydrolysis of panose (Fig. 3), glucose and small amounts of maltose were present, demonstrating the hydrolysis of the α-1,6 linkages. At complete hydrolysis, maltose disappeared and glucose

[6] The antibiotic mixture fed (36 mg. per pound of ration) was made up of equal parts of Terramycin, procaine penicillin, and streptomycin. We wish to thank Dr. A. H. Jensen of the Animal Science Department for making these animals available to us.

appeared as the only product. Maltase present in the extracts as a contaminant is responsible for the formation of glucose from maltose. In addition, faint spots appeared at late stages of the hydrolysis of panose (not visible in Fig. 3) which migrated at slower rates than isomaltose. These probably represent the result of transferring action taking place during the over-all hydrolysis, most likely oligosaccharides arising from maltase action. In maltose controls paper chromatographic analysis revealed, in addition to maltose and glucose, the presence of oligosaccharide spots at

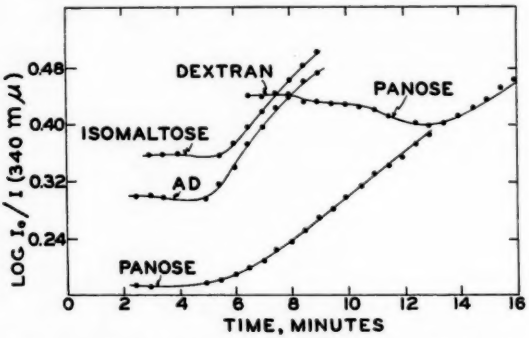


FIG. 4. Microenzymatic determination of oligo-1,6-glucosidase action on branched substrates. AD, α-amylase dextrins. Glycylglycine buffer 0.25 M, pH 7.4, 1.0 ml.; Mg^{++} 0.3 M, 0.1 ml.; *Zwischenferment*, 3 mg. of lyophilized powder per ml., 0.05 ml.; hexokinase, 2.8 per cent (20 per cent pure), 0.02 ml.; TPN 0.005 M, 0.1 ml.; ATP 0.1 M, 0.01 ml.; total volume 3.0 ml. The dextran reaction mixture contained 0.5 mg. of clinical dextran, 24 units of oligo-1,6-glucosidase, 0.5 mg. of panose; isomaltose reaction mixture, 0.4 mg. of isomaltose, 43.2 units of oligo-1,6-glucosidase; α-amylase dextrin reaction mixture, 0.058 mg. of α-amylase dextrins, 5.9 units of oligo-1,6-glucosidase; panose reaction mixture, 0.5 mg. of panose, 20 units of oligo-1,6-glucosidase.

intermediate stages of hydrolysis. Complete hydrolysis of the α-amylase dextrins gave glucose as the sole product.

Microenzymatic Identification of Glucose—Further identification of glucose as the reaction product of hydrolysis of branched saccharides has been provided by the microenzymatic test. Intestinal extracts have been coupled with the hexokinase, *Zwischenferment* system (21). As shown in Fig. 4, glucose produced from the hydrolysis of isomaltose, panose, or the α-amylase dextrins reacts in the microenzymatic test system with the rapid linear reduction of TPN. Under the conditions tested, the hog intestinal enzyme failed to liberate glucose from clinical dextran.[3, 7] Several points

[7] In another experiment, 21.6 units of oligo-1,6-glucosidase had no activity on 2 mg. of clinical dextran during an 11 minute time period.

merit further discussion. With weak oligo-1,6-glucosidase activity, there usually is an initial lag period of about 1 minute after substrate addition before TPN reduction begins. This is probably a function of the time required for saturation of hexokinase and *Zwischenferment* by the glucose and glucose-6-phosphate produced subsequent to the hydrolysis of the oligosaccharides. Intestinal extracts as freshly prepared contain large quantities of glucose which interfere in the microenzymatic determination. Dialysis against 0.85 per cent NaCl for 4 to 6 hours served to lower the glucose content to levels low enough so that there was no serious interference in the test. More highly purified glucosidase preparations contained no detectable glucose.

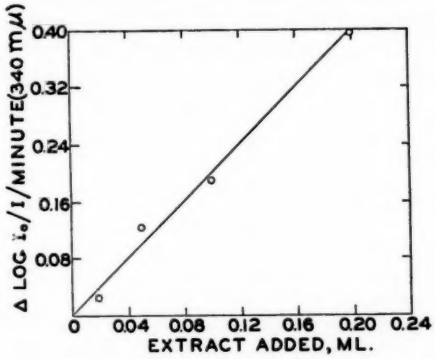


FIG. 5. Microenzymatic determination of oligo-1,6-glucosidase activity. The enzyme fraction contained 190 units per ml.; the other components were as described in Fig. 4.

Microenzymatic Assay and Purification—Fig. 5 provides the experimental basis for the microenzymatic assay system which has been adopted. A linear relationship between rate of TPN reduction and glucosidase added over a 10-fold range is obtained. Under these conditions, a unit of activity is defined as that amount of enzyme which catalyzes an increase in optical density of 0.001 per minute with isomaltose as substrate (0.0004 M). By the microenzymatic test, oligo-1,6-glucosidase activities of four species have been presented (6).

The enzyme from hog intestinal mucosa has been purified about 8-fold. A typical fractionation is presented in Table I.

Saturated ammonium sulfate (saturated at room temperature) at pH 7 was added to the extract to 0.35 saturation. After standing for 10 minutes at 0°, the heavy precipitate was removed by centrifugation. To the supernatant fluid, neutral saturated ammonium sulfate was again added to 0.80

saturation. After standing for 10 minutes at 0°, the precipitate was collected by centrifugation, dissolved in a minimal volume of 0.85 per cent NaCl, and dialyzed for 6 hours against a large volume of 0.85 per cent NaCl. This step usually results in about a 2-fold increase in specific activity with about a 60 to 80 per cent recovery. The protein concentration was determined (usually 2.5 per cent) and the reddish opalescent solution diluted to a protein concentration of 1 per cent. 0.2 ml. of alumina $C\gamma$ (22) (dry weight 14.8 mg. per ml.) was added per ml. of extract, and the mixture stirred for 5 minutes at 0°. After centrifugation, the slightly opalescent

TABLE I

Purification of Oligo-1,6-glucosidase

Fraction	Activity	Protein	Specific activity	Volume	Total units	Recovery
	units per ml.	*mg. per ml.*	*units per mg.*	*ml.*		*per cent*
Original extract	104	9.8	10.6	170.0	17,680	
Ammonium sulfate (0.35–0.80 saturation)	440	25.0	17.6	30.0	13,200	74.6
Supernatant after alumina adsorption	94	2.1	44.7	72.5	6,810	38.5
Ammonium sulfate (0–0.80 saturation)	320	9.4	34.0	12.5	4,000	22.6
Supernatant after calcium phosphate gel adsorption*	232	4.8	48.3	5.5	1,275	16.9
Ethanol fraction (18–44%)†	384	4.8	80.0	1.8	692	10.1

* An aliquot containing 1700 out of the 4000 units was used for the calcium phosphate gel adsorption. The recovery is calculated in terms of a recovery of 3000 units.

† An aliquot containing 1160 out of the 3000 units was used for the ethanol fractionation. The recovery is calculated in terms of a recovery of 1790 units.

supernatant fluid was treated with saturated ammonium sulfate (as above) to a concentration of 0.8 saturation. The precipitate was collected by centrifugation, dissolved in a minimal volume of 0.85 per cent NaCl, and dialyzed for 6 hours against 0.85 per cent NaCl.

The alumina adsorption step results in an additional 2- to 3-fold increase in specific activity with an over-all recovery of about 35 to 40 per cent. The enzyme is unstable in the dilute protein solution which remains after the alumina adsorption and is therefore concentrated with ammonium sulfate. Further loss in activity occurs during this latter step.

Additional purification has been achieved through the use of calcium phosphate gel adsorption and ethanol fractionation. The calcium phosphate gel adsorption is carried out after a preliminary dilution to a protein concentration of 1 per cent. 0.2 ml. of gel (23) (dry weight 10.4 mg. per

ml.) was added per ml. of extract, stirred for 5 minutes at 0°, and centrifuged. The supernatant fluid was then treated with 95 per cent ethanol at a temperature of from 0° to −5° and the fraction between 18 and 44 per cent ethanol collected by centrifugation, dissolved in a small volume of 0.85 per cent NaCl, and dialyzed for 2 hours against 0.85 per cent NaCl.

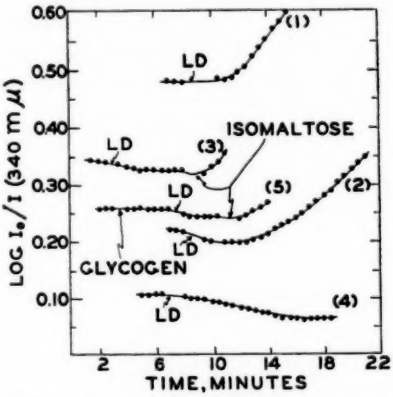


FIG. 6. Action of intestinal extracts on branched saccharides. Substrate additions were made at the times indicated; LD, muscle phosphorylase limit dextrin. Curve 1, initial extract. The initial extract reaction mixture contained 0.4 mg. of phosphorylase limit dextrin and 19 units of oligo-1,6-glucosidase. Curve 2, ammonium sulfate fraction (0.4 to 0.7 saturation). The ammonium sulfate fraction reaction mixture contained 1 mg. of phosphorylase limit dextrin and 23.4 units of oligo-1,6-glucosidase. Curve 3, ammonium sulfate fraction heated at 50° for 5 minutes. The heated ammonium sulfate fraction reaction mixture contained 1 mg. of phosphorylase limit dextrin, 0.4 mg. of isomaltose, and 21.2 units of oligo-1,6-glucosidase. Curve 4, ammonium sulfate fraction, aged at 3° for 8 days. The aged ammonium sulfate fraction reaction mixture contained 1 mg. of phosphorylase limit dextrin and 17.6 units of oligo-1,6-glucosidase. Curve 5, ammonium sulfate fraction treated twice with starch. The starch-treated ammonium sulfate fraction reaction mixture contained 1 mg. of phosphorylase limit dextrin, 0.4 mg. of isomaltose, 2.0 mg. of rabbit liver glycogen, and 19.4 units of oligo-1,6-glucosidase. All the other components were as described in Fig. 4.

The low over-all yields in the fractionation are due in the main to the steps in which the enzyme is precipitated from solutions of low protein concentration.

Differentiation from Amylo-1,6-glucosidase (24)—Intestinal extracts, before purification, liberate glucose from the muscle phosphorylase limit dextrin, Fig. 6 (Curve 1). This activity is lost under conditions which lead to purification of oligo-1,6-glucosidase. Ammonium sulfate fractionation (Curve 2) leads to a partial loss in activity, as evidenced by an increase in the length of time necessary to produce glucose from the mus-

cle phosphorylase limit dextrin. Further treatment, such as heat inactivation (Curve 3) or aging at 3° for 8 days (Curve 4), leads to a complete loss of activity under these test conditions. To decide whether the production of glucose from muscle phosphorylase limit dextrin was due to α-amylase (either acting alone or with oligo-1,6-glucosidase) or to amylo-1,6-glucosidase, extracts were treated with starch according to the method previously described (24). The loss in ability to form glucose from the muscle phosphorylase limit dextrin, or glycogen after two starch adsorptions (Curve 5), is taken as evidence that α-amylase is responsible for the glucose

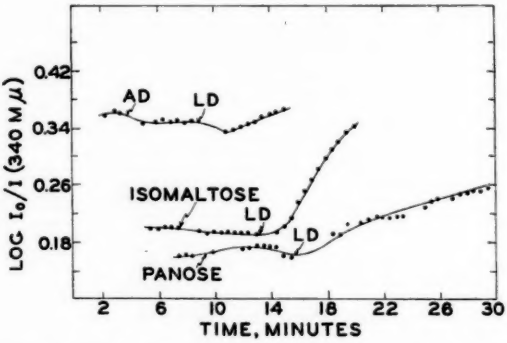


FIG. 7. Action of amylo-1,6-glucosidase on branched saccharides. LD, muscle phosphorylase limit dextrin; AD, α-amylase dextrins. The α-amylase dextrin reaction mixture contained 0.038 mg. of α-amylase dextrins, 1 mg. of phosphorylase limit dextrin, 264 units of amylo-1,6-glucosidase (24). The isomaltose reaction mixture contained 0.4 mg. of isomaltose, 1 mg. of muscle phosphorylase limit dextrin, 1116 units of amylo-1,6-glucosidase. The panose reaction mixture contained 1 mg. of panose, 1 mg. of phosphorylase limit dextrin, 375 units of amylo-1,6-glucosidase. All the other components were as described in Fig. 4.

released. A similar separation of α-amylase from α-limitdextrinase has been reported (7). These experiments indicate that oligo-1,6-glucosidase has, under these conditions, no activity on glycogen or on the muscle phosphorylase limit dextrin.

Amylo-1,6-glucosidase liberated no glucose under the experimental conditions of Fig. 7 from isomaltose, panose, or the α-amylase dextrins.[8] Sub-

[8] The present experiments do not rule out a very weak activity of amylo-1,6-glucosidase on the low molecular weight branched substrates such as the previously reported slow hydrolysis of isomaltose (24). Since muscle phosphorylase and amylo-1,6-glucosidase catalyze the essentially complete breakdown of glycogen and amylopectin, both enzymes must have some activity against low molecular weight branched substrates. The experiments of Fig. 7 were carried out in collaboration with Mr. L. H. Schliselfeld.

sequent addition of muscle phosphorylase limit dextrin led to rapid glucose production, indicating the activity of the test system.

TABLE II

Ratio of Maltase to Oligo-1,6-glucosidase Activity

Oligo-1,6-glucosidase fraction	Maltase activity (*1*)*	Oligo-1,6-glucosidase activity (*2*)	Ratio (*1*):(*2*)
	units per ml.	*units per ml.*	
Initial extract, 10 days at 3°	1920	296	6.5
Ammonium sulfate fraction (0.4–0.7 saturation)	4200	500	8.4
" " " incubated 5 min. at 50°	2210	288	7.7
Supernatant after 1 alumina adsorption†	1040	170	6.1
" " 2 " adsorptions	828	58	14.3
" " 1 calcium phosphate gel adsorption†	880	57	15.4
Supernatant after 2 calcium phosphate gel adsorptions	480	26	18.5

* The reaction mixture is the same as that for determination of oligo-1,6-glucosidase activity (Fig. 4), except that maltose was used as substrate.

† Alumina and calcium phosphate gel adsorptions were carried out as described in the text.

TABLE III

Stability of Oligo-1,6-glucosidase

	Activity	Recovery
	units per ml.	*per cent*
Control, pH 7.0	276	
pH 5.58, 60 min. at room temperature	110	39.8
" 9.77, 60 " " " "	148	53.6

Enzyme solutions were brought to the indicated pH's by the addition of 0.05 N HCl or 0.75 N NaOH. After standing for the indicated time, the solutions were neutralized to pH 7 with either solid $NaHCO_3$ or 2 N HCl, and tested microenzymatically. Two types of controls were run, with and without NaCl added in amounts equal to that present in the experimental vessels. There was no significant difference in these controls.

Separation of Oligo-1,6-glucosidase from Maltase—The early steps used in the purification of oligo-1,6-glucosidase (Table II) do not effect a separation of oligo-1,6-glucosidase from maltase. The ratio of maltase to oligo-1,6-glucosidase activities in the microenzymatic test system remains relatively constant (6.1 to 8.4). Separation of the two activities may be achieved by the selective adsorption of oligo-1,6-glucosidase on either alumina C_γ or

calcium phosphate gel. Under these conditions, the ratio of the two activities was increased[9] to 18.5. Seiji (7), fractionating with acetone and ammonium sulfate, was unable to separate these two activities.

Stability and pH Optimum—The crude enzyme is stable[10] for about 3 weeks at 3°. After this time, activity is decreased. A decrease in glucosidase activity of initial extracts is also brought about by storing in the frozen state. More highly purified preparations are stable in the frozen state. Lyophilization from either 0.85 per cent NaCl or glycylglycine buffer (0.025 M, pH 7.5) resulted in a loss of about 50 per cent of activity.

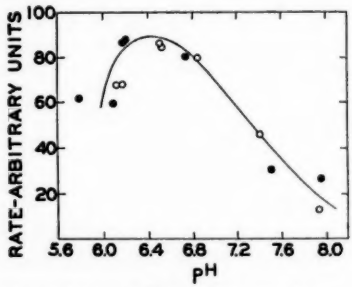


FIG. 8. pH-activity dependence of oligo-1,6-glucosidase. ○, microenzymatic determination; ●, determination by increase in reducing power; pH, that of the reaction mixture after incubation. The reaction mixtures for activity determination by increase in reducing power contained glycylglycine buffer 0.14 M; isomaltose, 0.8 mg.; oligo-1,6-glucosidase, 68 units; final volume 1.0 ml. The units represent Klett-Summerson colorimeter units. Microenzymatic reaction mixtures were as previously described (Fig. 4); highest activities were arbitrarily set equal to highest reduction units.

The enzyme is inactivated to a greater extent at acid than at alkaline pH (Table III).

The pH-dependence of oligo-1,6-glucosidase with isomaltose as substrate in glycylglycine buffer has been determined by both the microenzymatic method and the increase in reducing power. Maximal activity (Fig. 8) was found between pH 6.2 and 6.4. This agrees with Seiji's value of 6.2. Inhibition of oligo-1,6-glucosidase on the acid side of the pH curve was noted in acetate (0.14 M) and glycerophosphate (0.05 M) buffers.

[9] In a recent experiment a preparation of intestinal maltase free of detectable oligo-1,6-glucosidase activity has been prepared by fractionation of an acetone powder of hog intestinal mucosa.

[10] In some cases on standing at 3° for 2 to 3 days, there occurs an increase in oligo-1,6-glucosidase activity with no detectable change in protein concentration. This accounts for the 3-fold difference in specific activities of initial extracts reported in Table I and that previously reported (6). This activation process is under current investigation.

DISCUSSION

The evidence presented in the preceding sections establishes the presence of a new enzyme oligo-1,6-glucosidase in extracts of small intestinal mucosa. Oligo-1,6-glucosidase is involved in the terminal stages of starch digestion in the small intestine. Acting together with α-amylase and maltase, oligo-1,6-glucosidase catalyzes the essentially complete breakdown of starch to glucose in digestion. That this latter enzyme is of physiological significance in the whole animal is shown by the fact that isomaltose in the rat gives rise to as large an increase in liver glycogen as does maltose.[11]

Purified oligo-1,6-glucosidase from the hog does not liberate glucose from clinical dextran, glycogen, or the muscle phosphorylase limit dextrin under the conditions tested. It is therefore differentiated from amylo-1,6-glucosidase, which in turn has essentially no activity on the low molecular weight substrates. A partial separation from maltase has been achieved by differential adsorption on alumina Cγ, and calcium phosphate gel.

Seiji (7) has presented evidence that the enzymatic hydrolysis of α-amylase dextrins is catalyzed by an enzyme tentatively called α-limit-dextrinase, obtained from bovine intestinal mucosa and intestinal juice. The α-amylase dextrins which he prepared may have contained maltose (R_F of maltose 0.26, R_F of one spot in dextrin mixture 0.23) and are therefore subject to hydrolysis by the maltase present in the extracts.

Other plant, fungal, and bacterial α-1,6-splitting enzymes may now be mentioned. Oligo-1,6-glucosidase may be differentiated from R enzyme by the inability of the latter to hydrolyze isomaltose and panose (1). Limitdextrinase, an enzyme with similar activity to oligo-1,6-glucosidase from *Aspergillus oryzae* culture filtrates, has been reported (25). Similar hydrolysis of isomaltose and panose by mold culture filtrates has been reported by Pan and coworkers (26) and by Tsuchiya and coworkers (27). It is not known at present whether amyloglucosidase (γ-amylase) (28–30) or the maltase of *Clostridium acetobutylicum* (31) hydrolyzes α-1,6 linkages in addition to α-1,4.

SUMMARY

The hydrolysis of the α-1,6 linkages of isomaltose, panose, and α-amylase dextrins by an enzyme from intestinal mucosa called oligo-1,6-glucosidase has been demonstrated. Hydrolysis has been followed by increase in reducing power, paper chromatography, and by coupling to a specific micro-enzymatic test system.

Oligo-1,6-glucosidase has essentially no activity against the muscle phosphorylase limit dextrin. It is thus differentiated from amylo-1,6-glucos-

[11] Personal communication from Dr. R. C. Corley, Department of Chemistry, Purdue University, Lafayette, Indiana.

idase, which in turn has essentially no activity against isomaltose, panose, or the α-amylase dextrins. Partial purification and separation of oligo-1,6-glucosidase from α-amylase and maltase have been achieved.

Acting together with maltase, oligo-1,6-glucosidase catalyzes the complete hydrolysis of α-amylase dextrins and thus allows essentially complete digestion of starch to proceed in the gastrointestinal tract.

BIBLIOGRAPHY

1. Whelan, W. J., *Biochem. Soc. Symposia*, **11,** 17 (1953).
2. Nordin, P., Wild, G., and French, D., Abstracts, American Chemical Society, 124th meeting, Chicago, 53C, Sept. (1953).
3. Whelan, W. J., and Roberts, P. J. P., *Nature*, **170,** 748 (1952).
4. Meyer, K. H., and Gonon, W. F., *Helv. chim. acta*, **34,** 308 (1951).
5. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Interpretations, Baltimore, 2nd edition, **1** (1946).
6. Larner, J., and McNickle, C. M., *J. Am. Chem. Soc.*, **76,** 4747 (1954).
7. Seiji, M., *J. Biochem.*, Japan, **40,** 519 (1953).
8. Kornberg, A., *J. Biol. Chem.*, **182,** 805 (1950).
9. Larner, J., *J. Biol. Chem.*, **212,** 9 (1955).
10. Kornberg, A., and Horecker, B. L., *Biochem. Preparations*, **3,** 24 (1953).
11. French, D., Knapp, D. W., and Pazur, J. H., *J. Am. Chem. Soc.*, **72,** 5150 (1950).
12. Jeanes, A., Wise, C. S., and Dimler, R. J., *Anal. Chem.*, **23,** 415 (1951).
13. Hough, L., *Nature*, **165,** 400 (1950).
14. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
15. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100,** 695 (1933).
16. Somogyi, M., *J. Biol. Chem.*, **160,** 69 (1945).
17. Hers, H. G., and Kusaka, T., *Biochim. et biophys. acta*, **11,** 427 (1953).
18. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135,** 727 (1940).
19. Burton, R. M., and Stadtman, E. R., *J. Biol. Chem.*, **202,** 873 (1953).
20. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
21. Slein, M. W., *J. Biol. Chem.*, **186,** 753 (1950).
22. Wilstätter, R., Kraut, H., and Erbacher, O., *Ber. chem. Ges.*, **58,** 2448 (1925).
23. Keilin, F. R. S., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124,** 397 (1938).
24. Cori, G. T., and Larner, J., *J. Biol. Chem.*, **188,** 17 (1951).
25. Underkofler, L. A., and Roy, D. K., *Cereal Chem.*, **28,** 18 (1951).
26. Pan, S. C., Nicholson, L. W., and Kolachov, P., *Arch. Biochem. and Biophys.*, **42,** 406 (1953).
27. Tsuchiya, H. M., Montgomery, E. M., and Corman, J., *J. Am. Chem. Soc.*, **71,** 3265 (1949).
28. Corman, J., and Langlykke, A. F., *Cereal Chem.*, **25,** 190 (1948).
29. Kerr, R. W., Cleveland, F. C., and Katzbeck, W. J., *J. Am. Chem. Soc.*, **73,** 3916 (1951).
30. Phillips, L. L., and Caldwell, M. L., *J. Am. Chem. Soc.*, **73,** 3559 (1951).
31. French, D., and Knapp, D. W., *J. Biol. Chem.*, **187,** 463 (1950).

EFFECT OF FLUORIDE AND DINITROPHENOL ON ACETATE ACTIVATION IN KIDNEY AND LIVER HOMOGENATES*

By A. C. AISENBERG,† and VAN R. POTTER

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisconsin)

(Received for publication, December 18, 1954)

The past decade and a half has been marked by important advances in the understanding of the significance of acetate in intermediary metabolism (1–5). Though much has been learned of its importance in normal mammalian tissue, the rôle of acetate in tumor metabolism remains disputed. It is felt by some that neoplastic tissue is comparable to normal tissue in its ability to activate acetate (6, 7). However, there is considerable evidence that many tumors are deficient in the acetate-activating system or that conditions for acetate activation are unfavorable in these tumors (8–11). It is with the hope of eventually clarifying the problem of acetate activation in tumor tissue that these investigations of acetate activation in normal kidney and liver were undertaken.[1] The present studies concern the effect of fluoride and dinitrophenol on acetate activation in whole homogenates and mitochondrial preparations examined by means of carboxyl-labeled sodium acetate. Effects of these inhibitors have previously been studied in this laboratory in similar preparations with pyruvate and fumarate as substrates (13). That concentrations of fluoride as low as 0.0005 M might block acetate activation was previously indicated by its effect on octanoic acid activation ((14) foot-note 2). The present report is concerned with the localization of the fluoride effect and shows that the block is concerned with the primary activation of acetate rather than with steps that occur subsequent to the formation of acetyl coenzyme A or its hypothetical equivalent.

EXPERIMENTAL

The conventional Warburg apparatus was used. Male rats weighing from 250 to 300 gm. were obtained from the Holtzman Rat Company, were

* This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, Public Health Service, and in part by an institutional research grant (No. 71) from the American Cancer Society.

† Fellow in Cancer Research of the American Cancer Society.

[1] Under the conditions so far employed, homogenates of several transplantable rat tumors have shown negligible acetate activation and hence cannot be used to study the mechanism. The procedure used to demonstrate pyruvate oxidation in such homogenates (12) cannot be used in this situation because fluoride blocks acetate activation, as will be seen in this paper.

killed by decapitation and were bled, and the desired tissue was removed and chilled in isotonic sucrose solution (0.250 M) maintained at 0°.

Homogenate Experiments—Homogenates were prepared with a glass Potter-Elvehjem homogenizer. A 10 per cent homogenate was prepared in ice-cold isotonic sucrose, the general experimental method being similar to that of Pardee, Heidelberger, and Potter (8). Flasks were run in duplicate in all experiments with air as the gas phase; total fluid volume, 3.0 ml. Each flask contained the following, final flask concentrations being given in parentheses: 0.3 ml. of 0.1 M KH_2PO_4 (0.01 M), 0.4 ml. of 0.03 M $MgCl_2$ (0.004 M), 0.3 ml. of 0.01 M dipotassium salt of adenosine triphosphate (ATP) (0.001 M), 0.2 ml. of 0.03 M sodium acetate-1-C^{14} (0.002 M and 4200 c.p.m.[2] per flask), 0.03 to 0.15 ml. of 0.1 M potassium fumarate and potassium pyruvate (0.001 to 0.005 M). The final pH was adjusted to between 7.2 and 7.4 with 2 N KOH, a spot plate and phenol red being used as the indicator. Total osmolarity was adjusted to 250 milliosmoles with solid sucrose. Experiments were 50 to 70 minutes in duration and reactions were run at 38°.

Determination of Acetate Activation—Acetate activation was determined by two procedures. With small substrate pools, trapping (dilution by metabolic pools) was not important, and acetate activation was measured by the amount of $C^{14}O_2$ in the center well of the Warburg vessel (8).[2] In the presence of large substrate pools, trapping in the Krebs cycle intermediates invalidated the determination of center well radioactivity as the sole measure of acetate activation, and therefore residual non-volatile radioactivity of the flask contents was determined by a modification of the method used earlier (10). The perchloric acid filtrate was neutralized with 2 N KOH, and the resulting perchlorate removed by centrifugation after standing overnight in the refrigerator. A 0.7 ml. aliquot of the supernatant fluid from the perchlorate was acidified with 0.12 ml. of concentrated hydrochloric acid, 10 mg. of unlabeled sodium acetate were added, and the aliquot was evaporated to dryness in a desiccator attached to a water pump. The sucrose in the residue is partly carbonized. To insure complete removal of all radioactive acetate, the entire desiccation procedure was repeated. The residue after the second desiccation was resuspended in 0.5 ml. of water, and two 0.2 ml. aliquots were taken for plating on aluminum disks to determine the non-volatile radioactivity.[2] This radioactivity is largely in the Krebs cycle intermediates (9, 11).

Materials—Sodium acetate-1-C^{14} was prepared by Dr. H. Busch by the method of Lemmon (15). Carboxyl- and carbonyl-labeled pyruvic acids

[2] The counting was done in internal gas flow proportional counters under the supervision of Dr. Charles Heidelberger of this department. This skilled assistance is gratefully acknowledged.

were prepared from pyruvamide by hydrolysis and chromatographic purification on Dowex 1.[3] Redistilled commercial pyruvic acid was prepared by Dr. G. A. LePage and stored as a 1.0 N solution in the cold. Adenosine triphosphate was obtained as the dipotassium salt from the Pabst Laboratories, and other compounds were commercial products of reagent grade.

Miscellaneous—Cell fractionation was carried out in isotonic sucrose by the method of Schneider and Hogeboom (17). Adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate were determined by the method of Siekevitz and Potter (18).

Results

Preliminary Studies

Substrate Effect—Table I gives the results of variations of the amount of substrate upon oxygen uptake and acetate activation as judged by per cent of total C^{14} in the $C^{14}O_2$ in the center well. It is clear that the addition of either fumarate or glutamate to pyruvate as substrate leads to a marked increase in both oxidative rate and acetate activation (19). It is to be expected that the maximal yield of $C^{14}O_2$ would be the result of two opposing factors: (*a*) The yield is increased by increasing the substrate concentration at all points in the citric acid cycle to a level that achieves the maximal oxidative rate and presumably the maximal yield of the ATP necessary for the activation of acetate; and (*b*) the yield is decreased by any further increases in substrate concentration at any stage because the radioactivity of the active acetate is trapped or diluted in pools of Krebs cycle intermediates that are not converted to CO_2 in the experimental period. Moreover, the yield is influenced by the further fact that, as soon as the substrate level drops to a critical point, the yield of ATP becomes too low to maintain the integrity of the oxidative systems, and the system becomes inactive with the radioactivity of the activated acetate trapped permanently in the pools of intermediates (8). These effects had to be separated in order to localize the action of fluoride and of dinitrophenol.

Trapping Effect—Fig. 1 shows the effect of increasing concentrations of the combination of pyruvate plus fumarate (*cf.* Table I). It may be observed that, as the yield of radioactive CO_2 decreases beyond the maximum, the total yield of activated acetate continues to increase and reaches a plateau at approximately 100 per cent of the added acetate when the flasks contain 6 μmoles each of pyruvate and fumarate. It is clear that the

[3] The carboxyl-labeled pyruvamide was a gift from Dr. D. P. Groth and the carbonyl-labeled pyruvamide from Dr. Charles Heidelberger. The hydrolysis and purification of the resulting pyruvic acids were carried out with the help of Dr. D. P. Groth (16).

TABLE I

Effect of Substrate Levels on $C^{14}O_2$ in Center Well and on Oxygen Uptake

Each flask contained 40 mg. of whole kidney homogenate, 6 μmoles of acetate-1-C^{14} with a radioactivity of 4200 c.p.m., and other substrates as indicated. The results are tabulated as the per cent of original C^{14} that appeared in the $C^{14}O_2$ of the center well, and as the total oxygen uptake from 10 to 50 minutes.

Substrate			$C^{14}O_2$	Oxygen uptake
Pyruvate	Fumarate	Glutamate		
μmoles	*μmoles*	*μmoles*	*per cent*	*μl.*
			1.1	18
	3		3.5	87
	6		7.6	183
	12		1.9	182
3			3.9	68
6			3.9	59
1	1		5.3	74
3	3		33.0	276
6	6		16.9	332
3		3	8.6	223
3	3	3	26.5	319

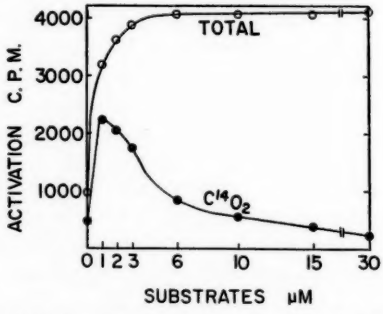


FIG. 1. Effect of substrate pool size on the trapping of radioactivity of acetate-1-C^{14}. Each flask contained 40 mg. of whole kidney homogenate and standard additions plus potassium pyruvate and fumarate, each in the amounts indicated along the abscissa, and also 6 μmoles of sodium acetate-1-C^{14} with a total radioactivity of 4200 c.p.m. The lower curve represents the $C^{14}O_2$ in the center well of the Warburg flask; the upper curve the total acetate activation; *i.e.*, the sum of the $C^{14}O_2$ in the center well and the residual non-volatile radioactivity of the flask.

optimal conditions for studying acetate activation in this complex system must involve the use of high enough concentrations of pyruvate and fumarate (15 μmoles each) to maintain the activation of acetate at a maximal linear rate, and that true comparison of inhibited rates and control rates

can be made only during the time intervals at which the rate in the control flasks is linear. However, considerable information can be gained from $C^{14}O_2$ output alone if the pyruvate and fumarate concentrations are at a suitable level (1 to 3 μmoles each), and if direct comparisons are made with labeled pyruvate as will be shown.

Intracellular Localization—The results of cell fractionation studies are presented in Table II, which contains data for liver and kidney. In the kidney experiment, acetate activation in the isolated mitochondria was equal to that of the reconstituted homogenate within the error of the method. For liver, only 55 per cent of the activity of the reconstituted homogenate was obtained from the mitochondrial fraction, but addition of the nuclear fraction increased this to 88 per cent. Other experiments

TABLE II

Intracellular Localization of Acetate Activation

Fractionation was performed in cold isotonic sucrose by the method of Schneider and Hogeboom (17). In the kidney experiment, each flask contained 40 mg. of kidney homogenate or equivalent cell fractions. In the liver experiment, each flask contained 80 mg. of liver homogenate or equivalent cell fractions. All the flasks contained 15 μmoles of pyruvate, 15 μmoles of fumarate, and 6 μmoles of acetate-1-C^{14}. The results are given as total activation in counts per minute per flask, *i.e.* the sum of $C^{14}O_2$ in the center well and residual non-volatile radioactivity of the flask.

	Whole homogenate	Reconstituted homogenate	Mitochondria	Mitochondria and nuclei	Mitochondria and supernatant fluid
Kidney	3850	3100	3100	3840	2720
Liver	2650	1890	1025	1650	1025

have shown that kidney mitochondria contain from 73 to 98 per cent of the total activity of the reconstituted homogenate, and liver mitochondria contain from 55 to 72 per cent. These data were obtained in systems that were not fortified with exogenous coenzyme A (CoA). Studies to be reported later indicate that the addition of CoA stimulates acetate activation in homogenates but not in mitochondrial preparations. The occurrence of a complete acetate-activating system in the mitochondria is in harmony with other studies (20–26), and shows that the system can be studied in mitochondria alone. The latter fact has certain advantages, particularly in the case of studies with dinitrophenol.

Studies with Fluoride and Dinitrophenol

Effect of Fluoride—Sodium fluoride was found to inhibit the activation of acetate in kidney and liver homogenates at concentrations of 0.0001 M and above. Fig. 2 illustrates this phenomenon in kidney homogenate at

high substrate concentration (15 μmoles each of pyruvate and fumarate). Acetate activation is plotted both as $C^{14}O_2$ and total activation, *i.e.* the sum of $C^{14}O_2$ and the residual non-volatile radioactivity. It can be seen that, at a fluoride concentration of 0.0003 M, acetate activation is depressed, while oxygen uptake is the same as in the non-fluoride-containing control. In other experiments, there was a significant depression of acetate activation at fluoride concentrations of 0.0001 M. It will also be noted

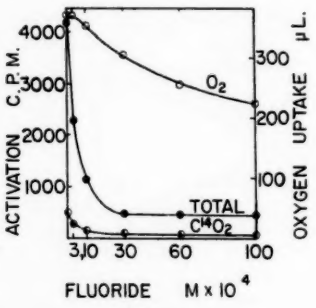


FIG. 2

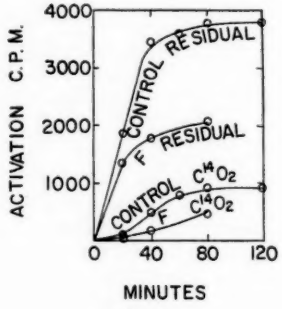


FIG. 3

FIG. 2. Effect of sodium fluoride on acetate activation and oxygen uptake. Each flask contained 40 mg. of whole kidney homogenate, 15 μmoles each of pyruvate and fumarate, and 6 μmoles of acetate-1-C^{14} with a radioactivity of 4200 c.p.m. as well as the standard additions. The acetate activation is plotted both as $C^{14}O_2$ in the center well and as total activation; *i.e.*, the sum of the $C^{14}O_2$ and the residual non-volatile radioactivity of the flask.

FIG. 3. Effect of sodium fluoride on acetate activation. The control flasks contained the same additions as in Fig. 2. The fluoride flasks contained a final fluoride concentration of 0.0003 M. Each point on the control and the fluoride curves indicates a duplicate pair of flasks in which the reaction was terminated at the appropriate time by tipping 0.3 ml. of 50 per cent perchloric acid from the side arm into the reaction mixture. Both residual non-volatile radioactivity of the flask and $C^{14}O_2$ in the center well are plotted for the control and fluoride flasks.

that increasing the fluoride concentration beyond 0.003 M does not lead to further depression of acetate activation. Even at a fluoride concentration of 0.01 M, 10 per cent of the activation of the control flasks is still present. Fig. 3 represents an experiment with kidney homogenate also performed at high substrate concentration. All flasks contained a final fluoride concentration of 0.0003 M, and it can be seen that this concentration markedly inhibits acetate activation. Further, it is apparent from Table III, in which the final amounts of adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate and the oxygen uptake are recorded for a similar experiment, that this effect of fluoride upon acetate

activation can occur without alteration in oxygen uptake or in the relative amounts of adenine nucleotides in the medium.

It should be stated parenthetically that Von Korff has found that sodium ions inhibit acetate activation (27). However, inhibition by sodium ions could hardly be involved in the inhibitory effect of sodium fluoride, since we obtained effects at 0.0001 M while Von Korff found that a 0.01 M sodium ion concentration caused only minimal depression of citrate formation.

TABLE III

Effect of Fluoride on Adenine Nucleotides and Oxygen Uptake

Each flask contained 40 mg. of whole kidney homogenate, 15 μmoles of pyruvate, 15 μmoles of fumarate, and 6 μmoles of acetate-1-C^{14} with a radioactivity of 4200 c.p.m. Duplicate experiments with and without fluoride were terminated at 20, 40, and 80 minutes by tipping 0.3 ml. of 50 per cent perchloric acid from the side arm into the reaction mixture. AMP, ADP, and ATP were determined by anion exchange chromatography on Dowex 1 by the method of Siekevitz and Potter (18). Fluoride-containing flasks had a fluoride concentration of 0.0003 M, and each flask contained 3 μmoles of adenosine triphosphate at the onset of the reaction. Oxygen uptake is given as the total uptake in microliters from 10 minutes after the flasks were placed in the bath to the termination of the experiment. Adenine nucleotides are expressed in micromoles.

	20 min.		40 min.		80 min.	
	Control	Fluoride	Control	Fluoride	Control	Fluoride
AMP	0.39	0.46	0.36	0.39	0.27	0.18
ADP	1.03	1.05	0.97	1.00	0.23	0.22
ATP	1.18	1.26	0.90	0.91	0.26	0.25
O_2 uptake	78	73	236	224	366	376

Parallel Studies on Acetate Activation and Pyruvate Oxidation—The depression of acetate activation caused by fluoride was compared with the effect of the same concentrations of fluoride on pyruvate oxidation. Experiments were carried on with pairs of duplicate flasks at each fluoride concentration. One pair of flasks had acetate-1-C^{14} and unlabeled pyruvate, while the other contained pyruvate-2-C^{14} and unlabeled acetate. The duplicate pairs were otherwise completely identical as to flask content. Fig. 4 represents a plot of two experiments performed in this manner with kidney homogenate. The curves for acetate and pyruvate oxidation are quite different, and the conclusion appears to be justified that the action of fluoride upon acetate oxidation is not the result of an inhibition of a reaction involved in pyruvate oxidation. Fig. 5 shows a similar experi-

ment with liver homogenate, and in this system it can be seen that, at fluoride concentrations of 0.0003 M and 0.001 M, inhibition of acetate oxidation is obtained without any effect on pyruvate oxidation.

Parallel Studies with Carbonyl- and Carboxyl-Labeled Pyruvate—An attempt was next made to clarify the cause of the minimal depression of pyruvate oxidation noted in kidney homogenates at low concentrations of fluoride. It was considered likely that this depression was secondary to the depression of acetate activation caused by fluoride. If the labeled

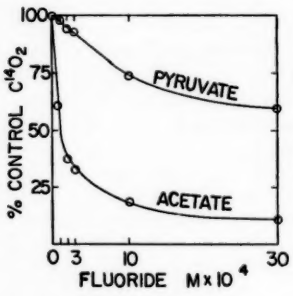


FIG. 4

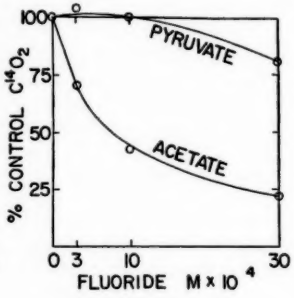


FIG. 5

FIG. 4. Parallel studies of the effect of fluoride on acetate and pyruvate oxidation. Each flask contained 40 mg. of whole kidney homogenate plus standard additions, and 3 μmoles of pyruvate and of fumarate. In addition, flasks represented by the pyruvate curve contained 6 μmoles of unlabeled acetate and pyruvate-2-C^{14} with a radioactivity of 4100 c.p.m. in less than 0.01 μmole. Flasks represented by the acetate curve contained 6 μmoles of acetate-1-C^{14} with a radioactivity of 4200 c.p.m. and unlabeled pyruvate. Pyruvate and acetate flasks were otherwise identical at each fluoride concentration. The results are plotted as the per cent of the $C^{14}O_2$ of controls with no fluoride added.

FIG. 5. Parallel studies of the effect of fluoride on acetate and pyruvate oxidation in liver. The conditions are the same as those of Fig. 4, except that 80 mg. of whole liver homogenate were used instead of kidney homogenate.

pyruvate were converted to acetate, this compound, which would be carboxyl-labeled when pyruvate-2-C^{14} was used, would be prevented from being reactivated by the fluoride block. This possibility was tested with parallel flasks of pyruvate labeled in different positions. The results of an experiment with pyruvate-1-C^{14} and pyruvate-2-C^{14} in a kidney homogenate system are given in Fig. 6. These results indicate clearly that at low fluoride concentrations there is depression of pyruvate oxidation as measured by the $C^{14}O_2$ in the center well with the carbonyl-labeled compound, but not when the carboxyl-labeled material is used. This supports the above postulate, since the trapped acetate fragment should be labeled in the case of the pyruvate-2-C^{14} but not in the case of pyruvate-1-C^{14}.

Effect of Dinitrophenol (DNP) on Acetate Activation—Fig. 7 represents an experiment performed with whole kidney homogenate with parallel flasks of acetate-1-C^{14} and pyruvate-2-C^{14}, and is a plot of per cent of control $C^{14}O_2$ in the center well against the dinitrophenol concentration. It shows that at a dinitrophenol concentration of 3×10^{-5} M there is inhibi-

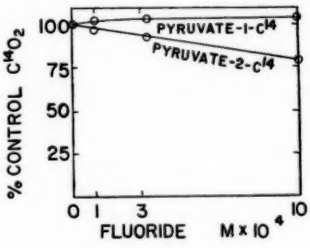


FIG. 6

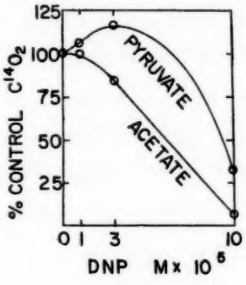


FIG. 7

FIG. 6. Parallel studies of the effect of fluoride on the oxidation of pyruvate-1-C^{14} and pyruvate-2-C^{14}. Each flask contained 40 mg. of whole kidney homogenate, standard additions, 3 μmoles of unlabeled pyruvate, 3 μmoles of unlabeled fumarate, and 6 μmoles of unlabeled acetate. In addition, at each fluoride concentration one pair of flasks contained pyruvate-1-C^{14} and one pair pyruvate-2-C^{14}. The labeled compound was added in amounts less than 0.01 μmole of pyruvate. The results are plotted as the per cent of the $C^{14}O_2$ in the center well of controls with no fluoride added.

FIG. 7. Parallel studies of the effect of dinitrophenol on acetate and pyruvate oxidation in whole kidney homogenate. Except for the use of dinitrophenol in place of fluoride, the conditions were identical with those of Fig. 4.

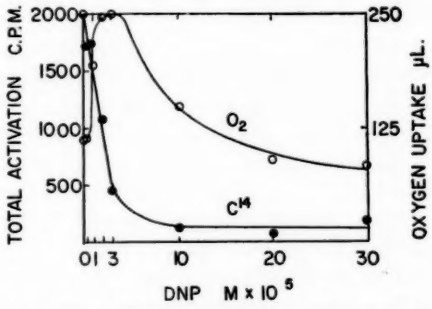


FIG. 8. Effect of dinitrophenol on acetate activation in rat liver mitochondria. Each flask contained the mitochondria from 80 mg. of liver. The substrates were 15 μmoles of pyruvate, 15 μmoles of fumarate, and 6 μmoles of sodium acetate-1-C^{14}. The results are plotted as total acetate activation, *i.e.* the sum of the $C^{14}O_2$ in the center well and the residual non-volatile radioactivity of the flask.

tion of acetate activation, while at the same time pyruvate oxidation is stimulated. Fig. 8 presents an experiment performed with isolated liver mitochondria at high substrate levels in which acetate activation was determined by the sum of center well radioactivity and residual nonvolatile radioactivity. The data are plotted as total acetate activation against dinitrophenol concentration. It can be seen that, at dinitrophenol concentrations up to 3×10^{-5} M, there is stimulation of oxygen uptake with simultaneous depression of acetate activation.

DISCUSSION

Known effects of fluoride on enzyme systems include inhibition of enolase (28), myokinase (29), phosphatases (30), cytochrome oxidase (31), succin-

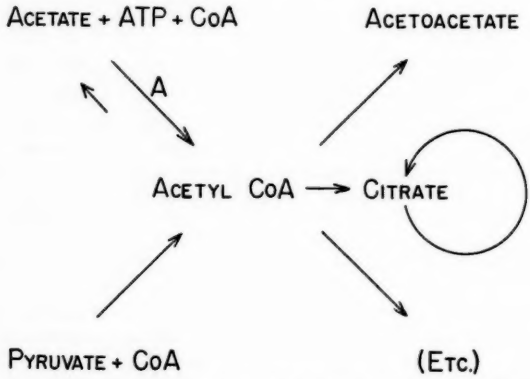


FIG. 9. Scheme of acetate activation. Modified from Chou and Lipmann (39)

oxidase (32), and nucleotide pyrophosphatases, including adenosinetriphosphatase (33). More closely related to the present work are the experiments of Johnson and Lardy with washed residue of rabbit liver homogenate (34). It was shown by these workers that a sodium fluoride concentration of 0.01 M prevented the enhancement of oxidative rate and inorganic phosphate uptake caused by the addition of acetate. Cheldelin and Beinert have found that sodium fluoride inhibits the oxidation of a number of fatty acids (35). Acetate was not studied by them.

The present studies are of interest in two regards. First, whereas in previous studies a fluoride concentration in the range of 0.01 M was used, in the present work inhibition of acetate activation was obtained with fluoride concentrations as low as 0.0001 and 0.0003 M. Secondly, these effects have been obtained without effect on oxidative rate or concentrations of adenine nucleotides in the medium, and particularly without effect

on the oxidation of pyruvate. Assuming a scheme such as that indicated in Fig. 9, an effect by fluoride on acetate activation without an effect on pyruvate oxidation would seem to involve specifically Reaction A, the formation of acetyl CoA from acetate, CoA, and adenosine triphosphate. For further consideration, the over-all reaction may be written as it appears in recent papers by Lipmann *et al.* (36–40), by Hele (24), and by Beinert *et al.* (41).

$$\text{Acetate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{pyrophosphate} \quad (1)$$

In examining the mechanism of this reaction, Lipmann *et al.* originally proposed (36) a two-step reaction as follows:

$$\text{ATP} + \text{CoA} \rightleftharpoons \text{AMP} + \text{CoA pyrophosphate} \quad (2)$$

$$\text{CoA pyrophosphate} + \text{acetate} \rightleftharpoons \text{acetyl CoA} + \text{pyrophosphate} \quad (3)$$

The sum of these two reactions would be the over-all Reaction 1. This mechanism implies the existence of two enzymes and the occurrence of CoA pyrophosphate. However, neither Jones *et al.* (38) nor Beinert *et al.* (41) found any further support for either of these two implications, and a new mechanism was proposed by the Lipmann group (37):

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{enzyme} \sim \text{AMP} + \text{pyrophosphate} \quad (4)$$

$$\text{Enzyme} \sim \text{AMP} + \text{CoA} \rightleftharpoons \text{enzyme} \sim \text{CoA} + \text{AMP} \quad (5)$$

$$\text{Enzyme} \sim \text{CoA} + \text{Ac} \rightleftharpoons \text{enzyme} + \text{acetyl CoA} \quad (6)$$

The sum of these reactions is the same over-all Reaction 1. This mechanism theoretically involves only one enzyme, and as such may be distinguished from the two enzyme theory. It might be thought that fluoride depression of acetate activation is secondary to fluoride depression of pyrophosphatase (*cf.* Reaction 4), but we have so far been unable to demonstrate either convincing accumulation of pyrophosphate or depression of acetate activation by pyrophosphate in the fluoride-inhibited system. Fluoride depression of acetate activation may be secondary to one of the other known effects of fluoride listed earlier, or to a new and as yet unobserved action of the compound. On the preliminary evidence now available, we would favor the latter explanation.

In the experiments with dinitrophenol, acetate activation is diminished at the lowest concentrations of dinitrophenol employed, 1×10^{-5} and 3×10^{-5} M. At these concentrations the rate of oxygen uptake is increased, and there is no decrease in the ATP level of the medium (42). However, DNP has been shown to lower the level of ATP and raise the level of AMP in the mitochondria (43). The effect of DNP on acetate activation may be purely a reflection of this effect. This would certainly

slow Reaction 1, while at the same time it would stimulate oxygen uptake. These are the results which we have observed experimentally (Fig. 7).

It may be noted that the Lipmann group in their studies on CoA reactions (36–39) have used from 0.02 to 0.05 M fluoride to inhibit pyrophosphate breakdown, while in the present work the presence of much smaller quantities produced profound inhibition of the over-all reaction. A possible explanation for their ability to obtain acetate activation in the presence of fluoride is that, in their studies, CoA was present in stoichiometric amounts, while in the present work the small amount of CoA originally present was continually regenerated by the further reaction of acetyl CoA with oxalacetate. We have found that fluoride inhibition is modified by the CoA concentration. Studies on this aspect of the problem are still in progress. Another possible basis for the differences in sensitivity to fluoride is the fact that the studies by the Lipmann group have been carried out with enzymes in solution, while the present work has been carried out with the system as it occurs in intact mitochondria.

SUMMARY

1. Acetate activation was studied in rat liver and kidney homogenates and mitochondrial preparations through the use of acetate-1-C^{14}, pyruvate-1-C^{14}, and pyruvate-2-C^{14}.

2. Concentrations of sodium fluoride as low as 0.0001 M were found to inhibit the activation of acetate, without affecting pyruvate oxidation, oxidative rate, or the level of the adenine nucleotides in the medium. It was suggested that fluoride acts on the formation of acetyl CoA from acetate.

3. Dinitrophenol was shown to depress acetate activation at levels which stimulated oxidative rate and pyruvate oxidation.

BIBLIOGRAPHY

1. Bloch, K., *Physiol Rev.*, **27,** 574 (1947).
2. Lipmann, F., *Harvey Lectures*, **44,** 99 (1948–49).
3. Lynen, F., *Harvey Lectures*, **48,** 210 (1952–53).
4. Ochoa, S., *Harvey Lectures*, **46,** 153 (1950–51).
5. Barker, H. A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 204 (1951).
6. Weinhouse, S., Allen, A., and Millington, R. H., *Cancer Res.*, **13,** 367 (1953).
7. Olson, R. E., *Cancer Res.*, **11,** 571 (1951).
8. Pardee, A. B., Heidelberger, C., and Potter, V. R., *J. Biol. Chem.*, **186,** 625 (1950).
9. Busch, H., *Cancer Res.*, **13,** 789 (1953).
10. Busch, H., and Potter, V. R., *Cancer Res.*, **13,** 168 (1953).
11. Busch, H., and Baltrush, H. A., *Cancer Res.*, **14,** 448 (1954).
12. Potter, V. R., and Lyle, G. G., *Cancer Res.*, **11,** 355 (1951).
13. Siekevitz, P., and Potter, V. R., *Cancer Res.*, **13,** 513 (1953).

14. Potter, V. R., *J. Biol. Chem.*, **169,** 17 (1947).
15. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., Isotopic carbon, New York (1949).
16. Groth, D. P., and LePage, G. A., *J. Am. Chem. Soc.*, **77,** 1681 (1955).
17. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183,** 123 (1950).
18. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **200,** 187 (1953).
19. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172,** 389 (1948).
20. Recknagel, R. O., and Potter, V. R., *J. Biol. Chem.*, **191,** 263 (1951).
21. Elliott, W. B., and Kalnitsky, G., *J. Biol. Chem.*, **186,** 477 (1950).
22. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **172,** 847 (1948).
23. Schneider, W. C., *J. Biol. Chem.*, **176,** 259 (1948).
24. Hele, P., *J. Biol. Chem.*, **206,** 671 (1954).
25. Mahler, H. R., Wakil, S. J.,and Bock, R. M., *J. Biol. Chem.*, **204,** 453 (1953).
26. Bartley, W., *Biochem. J.*, **53,** 305 (1953).
27. Von Korff, R. W., *J. Biol. Chem.*, **203,** 265 (1953).
28. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1942).
29. Barkulis, S. S., and Lehninger, A. L., *J. Biol. Chem.*, **190,** 339 (1951).
30. Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, **29,** 842 (1935).
31. Slater, E. C., *Biochem. J.*, **44,** 305 (1949).
32. Slater, E. C., and Bonner, W. D., Jr., *Biochem. J.*, **52,** 185 (1952).
33. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **182,** 763 (1950).
34. Johnson, R. B., and Lardy, H. A., *J. Biol. Chem.*, **184,** 235 (1950).
35. Cheldelin, V. H., and Beinert, H., *Biochim. et biophys. acta*, **9,** 661 (1952).
36. Lipmann, F., Jones, M. E., Black, S., and Flynn, R. M., *J. Am. Chem. Soc.*, **74,** 2384 (1952).
37. Jones, M. E., Lipmann, F., Hilz, H., and Lynen, F., *J. Am. Chem. Soc.*, **75,** 3285 (1953).
38. Jones, M. E., Black, S., Flynn, R. M., and Lipmann, F., *Biochim. et biophys. acta*, **12,** 141 (1953).
39. Chou, T. C., and Lipmann, F., *J. Biol. Chem.*, **196,** 89 (1952).
40. Lipmann, F., Jones, M. E., Black, S., and Flynn, R. M., *J. Cell. and Comp. Physiol.*, **41,** suppl. 1, 109 (1953).
41. Beinert, H., Green, D. E., Hele, P., Hift, H., Von Korff, R. W., and Ramakrishnan, C. V., *J. Biol. Chem.*, **203,** 35 (1953).
42. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **201,** 1 (1953).
43. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **215,** 237 (1955).

APPLICATION OF THE ISOTOPE DERIVATIVE METHOD TO THE ANALYSIS OF PYRIMIDINES*

By JACQUES R. FRESCO† and ROBERT C. WARNER

(From the Department of Biochemistry, New York University College of Medicine, New York, New York)

(Received for publication, February 2, 1955)

In recent years several methods have been introduced for the analysis of the nitrogenous constituents of nucleic acids (*cf.* (2, 3)). However, a specific method is lacking for accurate analysis of microgram quantities of these substances in hydrolysates of biological specimens from which the nucleic acids are not readily isolated. A method which fulfils this need is presented. It applies the principles of the *isotope derivative method* (4, 5) to the determination of uracil and thymine, the pyrimidines characteristic of ribonucleic (RNA) and deoxyribonucleic (DNA) acids, respectively. The isotope derivative method, as employed here, involves the quantitative conversion of uracil or thymine to isotopically labeled *p*-iodophenylsulfonyl (pipsyl) derivatives, which are then quantitatively determined by *carrier* (4) or *indicator* (5) procedures without the necessity for quantitative isolation of the derivatives.

EXPERIMENTAL

Reaction of Pyrimidines and Purines with Pipsyl Chloride

Pipsyl chloride was explored for use as the analytical reagent because it was expected to react with compounds which possess amino or hydroxyl groups and it can be labeled with two distinguishable isotopes, I^{131} and S^{35}. After examining a variety of aqueous and organic one- and two-phase reaction systems, it was found that uracil,[1] thymine, isocytosine, hypoxanthine, and 2,6-diaminopurine, but not 2-aminopyrimidine, cytosine, ade-

* A preliminary report of this work was presented at the Fortieth annual meeting of the American Society of Biological Chemists, April, 1949 (1). Taken, in part, from the thesis submitted by Jacques R. Fresco in May, 1952, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Science of New York University. This work was supported in part by grants to Dr. Milton Levy by the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. The iodine and sulfur isotopes used in this study were supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

† Present address, Department of Pharmacology, New York University College of Medicine, New York.

[1] Substituted pyrimidines are numbered so that uracil is 2,4-dihydroxypyrimidine.

XUM

nine, and guanine formed monopipsyl derivatives in good yield. It would appear, therefore, that only those pyrimidines and purines which possess potentially enolic groups with pK_a values in the region of 9 form those derivatives readily.[2] A one-phase 75 per cent acetone system at room temperature, buffered at pH 8.5 to 9.0 with tetramethylammonium bicarbonate, was found to be the most effective medium for reaction of pipsyl

TABLE I

Recovery Control Analyses for Uracil and Thymine

Pyrimidine	Method	Pyrimidine added	Pipsyl chloride* added	Pyrimidine found	Recovery
		μmoles	*μmoles*	*μmoles*	*per cent*
Uracil	Carrier	0.373		0.353	94.7†
		0.373		0.350	94.0†
		0.554	26.04	0.524	94.5
		0.745		0.702	94.2
		0.789	10.26	0.748	94.8
		0.793		0.740	93.2‡
		1.525		1.416	93.0
		1.525		1.463	95.8
		4.59		4.35	94.8
		4.59	27.5	4.29	93.5
		4.62	13.9	4.33	93.8
	Indicator	0.00378	0.15	0.00352	93.0
		0.745		0.709	95.0
Thymine	Carrier	0.550		0.518	94.1
		0.725		0.686	94.6
	Indicator	0.725		0.673	92.7

* Unless otherwise indicated, the amount of pipsyl chloride added was 5 to 10 times the amount of pyrimidine added.

† Uracil dissolved in 60 per cent $HClO_4$.

‡ Recovered as additional uracil when it was added to a nucleic acid hydrolysate previously analyzed.

chloride with uracil and thymine. A consistent 94 per cent conversion to monopipsyl derivatives has been obtained in recovery control analyses. This yield was not altered when the molar ratio of pipsyl chloride to pyrimidine was varied from 3 to 47 (Table I), indicating that dipipsyl derivatives are not formed.

The pipsylpyrimidines are stable at room temperature below pH 10; in stronger alkali they are readily hydrolyzed to *p*-iodophenylsulfonic acid (pipsyl acid) and the free pyrimidines. The derivatives are slightly soluble

[2] 2,6-Diaminopurine is an exception to this generalization for which there is no apparent explanation.

in acid and are soluble in alkali, alcohol, acetone, and ether. Some of their properties are presented in Table II.

Several considerations bear on the structure of these derivatives. (*a*) Nitrogen analyses are consistent with monopipsyl forms. (*b*) Coupling between pipsyl chloride and the pyrimidines does not take place below pH 8. (*c*) For uracil and thymine, dissociation occurs first at positions 1:2 (pK_{a_1} values 9.5 and 9.9, respectively) and then at positions 3:4 (pK_{a_2} values greater than 13) (6).[3] (*d*) In the acetone medium referred to above, 1-methyluracil[4] and 2-ethoxy-4-hydroxypyrimidine[4] did not form pipsyl derivatives, while 4-ethoxypyrimidone-2[4] did. Hydrolysis of the 4-ethoxy group from the pipsyl derivative of the latter in ethanolic HCl at room temperature yielded pipsyluracil. Thus, replacement of the dissociable proton of the 1:2 positions of uracil prevented reaction with pipsyl chloride, while corresponding replacement at the 3:4 positions did not. Therefore, attachment of the pipsyl moiety at the 3 or 4 position of these pyrimidines is eliminated. It is presumed that pipsyluracil and pipsylthymine are monosubstituted at the 1 or 2 position of the heterocyclic ring. The data do not permit a decision between these two positions. Benzenesulfonylpyrimidines were also prepared by treating uracil or thymine with benzenesulfonyl chloride in the acetone medium; they are presumed to be structural analogues of the pipsylpyrimidines.

TABLE II

Properties of Pipsyl Derivatives

Derivative	Decomposition point, uncorrected	E_m Wave-length maxima	N, found	N, theoretical	Crystal form
	°C.		*per cent*	*per cent*	
Pipsyluracil	234–239	20,700 in 0.04 N HCl at 260 mμ	7.41	7.40	Flat prisms
Pipsylthymine	229–231	20,230 in 0.04 N HCl at 264 mμ	7.16	7.15	Fine long needles

Preparation of Compounds

Non-Isotopic Pipsyluracil and Pipsylthymine Carriers—Uracil or thymine was treated with a 2- to 3-fold excess of pipsyl chloride (4) in the buffered acetone medium (see below) for 1 to 2 hours. The reaction mixture was then acidified and the acetone removed *in vacuo*. The resulting pre-

[3] The symbol 1:2 is used because the dissociating proton is involved in a lactam-lactim tautomerization and it has not been possible to determine the position from which it dissociates.

[4] These compounds were kindly furnished by Dr. Jack Fox.

cipitate was washed with water and recrystallized (several times from each solvent) with charcoal treatments, first from *ammoniacal-alcohol buffer* (0.36 M NH_3 and 0.12 M NH_4Cl in 80 per cent alcohol) by dilution with water and acidification, next from acetone, then from 95 per cent alcohol. It was finally washed with water and dried at 105°. The best evidence of the purity of these compounds is that the procedure described above yielded products of constant specific activity when a labeled reagent was employed (*cf.* "Carrier analysis"). It was further indicated by the reproducibility of nitrogen content and molar extinction coefficient observed in a number of preparations.

Uracil and Thymine Standards—Pure benzenesulfonyl or pipsyl derivatives of uracil or thymine were hydrolyzed in excess N alkali at 100°. The pyrimidines recovered on acidification of these hydrolysates were recrystallized several times from water with charcoal treatments and dried at 105°. The purity of these compounds was confirmed by determination of nitrogen content, molar extinction coefficient, and decomposition point.

S^{35}-Labeled Pipsyluracil and Pipsylthymine Indicators—To conserve S^{35}-labeled pipsyl chloride (5), a 3-fold excess of the pyrimidine (10 mg.) was treated in the acetone medium with the reagent (10 mg.). The acidified reaction mixture (see below) was extracted several times with ether. The pooled ether extracts were washed with water and extracted several times with equal volumes of borate buffer (pH 10); the pipsylpyrimidine, precipitated from the borate extracts on acidification, was washed with water and recrystallized several times from *ammoniacal-alcohol buffer*. The final precipitate was washed with water and dissolved in 50 per cent alcohol or 50 per cent acetone for use in the *indicator* analyses. The purity of an indicator was checked by paper chromatographic analysis (*cf.* "Indicator analysis").

Tetramethylammonium Bicarbonate Buffer—Carbon dioxide was bubbled through tetramethylammonium hydroxide (1.1 M) until the solution indicated a pH of about 8.5 with Hydrion paper. Occasionally a gelatinous precipitate formed which was removed by centrifugation.

Analytical Scheme for Uracil and Thymine

Earlier presentations of the isotope derivative method (4, 5, 7) should be consulted for more explicit details and discussion of the methods and calculations described here.

Reaction Mixture—The unknown is dissolved in a minimal volume of tetramethylammonium bicarbonate buffer or is brought to about pH 8.5 by the addition of tetramethylammonium hydroxide. To this solution are added pipsyl chloride in at least a 10-fold excess over the reactive groups presumed to be in the unknown, enough tetramethylammonium bicarbon-

ate buffer to neutralize the acid which may be formed during the reaction, and acetone to a final concentration of 75 per cent, after which the reaction vessel is stoppered. This reaction mixture is shaken to insure solution, kept at room temperature for 1 hour, and then acidified with HCl in 75 per cent acetone.

Carrier Analysis—I^{131}-labeled pipsyl chloride (4) is employed for the reaction. The acidified reaction mixture is quantitatively transferred to a vessel containing an accurately weighed amount (50 to 100 mg.) of the carrier of the compound of interest. Homogeneous distribution of the carrier in the reaction mixture is effected by adding 75 per cent acetone to the mixture and heating until all the carrier is dissolved. Most of the acetone is then evaporated and the carrier precipitates on cooling.

When uracil and thymine are both presumed present in the unknown, a preliminary separation of their derivatives is effected after addition of both

TABLE III

Distribution Coefficients

Expressed as the ratio of concentration in the organic phase to that in the aqueous phase.

Derivative	Ether and 0.1 M borax (pH 9.2)	Ether and 0.2 M borate buffer (pH 10.0)	Ether and 0.01 N HCl
Pipsyluracil	1.1	0.14	38
Pipsylthymine	6.2	1.1	66
Pipsyl acid			0.0

carriers by means of a modified seven plate counter-current distribution between 2 volumes of 0.1 M borax buffer (pH 9.2) and 1 volume of ether. This separation is based on the distribution coefficients given in Table III. The precipitated carriers are washed with water and dissolved in the ether phase of the first plate. Four aqueous phases are each completely transferred through the seven ether phases. The pooled ether phases of the first four plates, containing the bulk of the pipsylthymine, are extracted three times with 0.3 volume of 0.1 N NaOH. The alkaline extracts are pooled and *immediately* acidified to precipitate the pipsylthymine. The four aqueous phases are also pooled and the pipsyluracil therein precipitated by acidification.

The individual carriers are readily recrystallized in good yield from the *ammoniacal-alcohol buffer* by dilution with water and acidification with HCl to below pH 3. Generally, eight to ten recrystallizations of the carrier, with alternate charcoal treatments (Norit A), are sufficient for removal of coprecipitating pipsyl derivatives formed in reaction mixtures containing nucleic acid hydrolysis products (*cf.* Table V). A pure carrier

is indicated by constancy of specific activity[5] through a series of crystallizations. Typical examples of such data are presented in Table IV.

For determination of its specific activity,[5] the carrier is dissolved in 7 ml. of the *ammoniacal-alcohol buffer* and then diluted to 10 ml. with water. An aliquot of 100 to 200 μl. is accurately obtained with a microconstriction pipette (8) and diluted to a known volume with 0.04 M HCl. The optical density, measured against a suitable blank at the appropriate wave-length of maximal absorption, serves to determine the concentration of carrier.

TABLE IV

Specific Activities of Purified Carriers

The carriers were isolated from pipsylation reaction mixtures of nucleic acid hydrolysates.

Pipsyluracil			Pipsylthymine		
No. of crystallizations	Carrier remaining	Specific activity*	No. of crystallizations	Carrier remaining	Specific activity*
	per cent			*per cent*	
8	31	0.00370	6	40	0.00213
13	25	0.00374	10	30	0.00208
16	19	0.00372	13	24	0.00211
Average		0.00372			0.00211

* See foot-note 5.

Radioactivity can be measured in one of two ways. In one method, preferable when the specific activity of the isolated carrier is low, an accurately measured aliquot (3 to 10 ml.) of the ammoniacal-alcohol carrier solution is placed in an annular glass counting cell (4). After determining its radioactivity with a Geiger counter, the aliquot can be removed for further recrystallization. The measurable radiation of such liquid samples depends, in part, on the density (the salt and solvent concentration) of the sample. It is therefore necessary to dilute unknowns and reagent standards to the same concentration of *ammoniacal-alcohol buffer* and to take equal volumes of each for measurement of radioactivity. In the second method of radioactive analysis of the carrier solution an aliquot, the salt content of which is too low to incur an error because of self-absorption, *less* than 50 μl., is delivered onto a metal planchet already containing a 0.2 ml. drop of water. The sample is dried and counted under a thin mica window Geiger tube. The latter procedure is applicable only when the

[5] Isotope concentration or specific activity is defined as the ratio of counts per minute per micromole of sample to counts per minute per micromole of reagent standard.

specific activity of the carrier is so high that such a small sample can be taken for analysis. In either procedure an appropriate "reagent" standard consisting of a known amount of a pure pipsyl derivative, prepared with the same I^{131}-labeled pipsyl chloride that was used in the analysis, is counted at the beginning and end of each counting period. In addition to providing the specific activity of the reagent, this standard serves to correct for the decay of I^{131}.

TABLE V

Zero Control Analyses with Pipsyluracil Carrier

5 μmoles of the indicated substance were treated with I^{131}-pipsyl chloride in the acetone medium. 100 to 200 μmoles of carrier pipsyluracil were then added. Purification of the carrier was conducted as indicated.

Substance	Apparent uracil found		No. of recrystallizations
	μmole	*per cent of added substance*	
Adenine	0.0062	0.12	8
Guanine	0.0061	0.12	8
Xanthine	0.0085	0.17	14
Hypoxanthine	0.211	4.2	8
"	0.005	0.1	6*
Cytosine	0.0028	0.05	5
Thymine	0.04	0.8	22
"	0.022	0.4	5*
Protein hydrolysate	0.046	0.9	8
" "	0.0156	0.3	4*
I^{131}-Pipsyl acid†	0.0038	0.009	8

* Partially purified by counter-current distribution prior to recrystallization.
† 40.5 μmoles of pipsyl acid were used.

The amount of carrier added and the specific activities of the reagent and the pure isolated derivative are the data required to calculate the quantity of unknown (4).

In addition to the recovery control analyses (Table I) which checked the yield, the carrier procedure was tested for specificity in zero control analyses. The latter involved determinations of uracil on samples *lacking uracil* but containing a variety of possible contaminants. The samples were treated with I^{131}-pipsyl chloride. Pipsyluracil carrier was then added and purification was carried out by crystallization and counter-current procedures. In the examples presented in Table V, constant (zero) specific activity of the carrier had not yet been achieved at the time the analyses were terminated, but the amounts of carrier remaining were sufficient to have permitted many more recrystallizations.

Indicator Analysis—The unknown sample is treated with I^{131}-labeled pipsyl chloride reagent. The acidified reaction mixture is diluted to a known volume with 50 per cent acetone. To an aliquot of this dilution are added aliquots of the appropriate S^{35}-labeled indicators. The solution is warmed to evaporate the acetone, diluted with 0.1 N HCl to a convenient volume, and extracted twice with equal volumes of ether. The pipsylpyrimidines pass into the ether phase, leaving most of the pipsyl acid, a major contaminant, in the aqueous phase. The ether extracts are pooled, evaporated to a small volume, and an aliquot containing an appropriate number of counts is applied to a Whatman No. 1 filter paper strip for chromatography. The chromatogram is developed with the organic phase of a *freshly* equilibrated mixture of tetrachloroethylene, c.p. (1 volume), butanol (0.02 to 0.03 volume), and H_2O (1 volume), in which the retardation factors for pipsyl acid, pipsyluracil, and pipsylthymine are approximately 0.0, 0.5, and 0.7, respectively. The best resolution is achieved in this solvent when the amount of each derivative applied to a paper strip 3 cm. wide is less than 10 γ. It is sometimes desirable to remove a band and rechromatograph its eluate in the same solvent in order to remove tailing contaminants.

The developed paper chromatograms are air-dried and the components located by radioautography. The appropriate bands are cut out and divided into four or five transverse strips each. These are individually eluted with weak ammonia water or 50 per cent acetone, and the eluates are dried on metal planchets. The samples are almost weightless and are counted with a mica window Geiger tube without concern for self-absorption.

The radiation characteristics of I^{131} and S^{35} are distinguishable by differential penetration through thin aluminum filters. Each planchet is counted with ($\bar{c}$ counts) and without a filter ($\bar{s}$ counts), and the ratio of these respective counts, $\bar{c}/\bar{s}$, is compared for each of the several planchets made from the parallel strips in each band. If the ratios of consecutive bands show deviations from their average greater than predicted from the number of disintegrations measured, the band is considered impure. Examples of acceptable ratios are shown in Table VI. An I^{131} "reagent" standard (*cf.* "Carrier analysis") and an S^{35} "indicator" standard containing a known aliquot of the particular S^{35} indicator are also counted with and without a filter along with each set of planchets.

The filter factors for pure I^{131} and S^{35} ($\bar{c}/\bar{s}$ for "reagent" and "indicator" standards), the specific activity of the reagent, the number of counts of indicator added, and the $\bar{c}/\bar{s}$ ratio of the particular band are the data required to calculate the quantity of unknown (5).

Accuracy of Analyses—The consistent recoveries of uracil and thymine

(94 ± 1 per cent) obtained in the control analyses (Table I) justify the use of the correction factor 1/0.94. This has been applied to the analytical data obtained by either carrier or indicator procedures (Tables VII and VIII).

The accuracy of the analyses is limited primarily by the error in counting. In the carrier method, this error can be held to 1 per cent by determining 10,000 counts on samples in which the radioactivity is at least 10 times that of background. In the indicator analyses, the errors in several counts can combine to give a larger than additive over-all counting error. To prevent a total error due to counting of greater than 1 per cent it is therefore necessary to determine 20,000 to 40,000 counts on most samples. For a filter which holds back about 99 per cent of the S^{35} activity and 35

TABLE VI

Ratios of Counts Measured with and without Filter ($\bar{c}/\bar{s}$) of Eluates of Consecutive Strips of Pure Bands Obtained from Paper Chromatograms

The pipsyluracil and pipsylthymine were isolated from pipsylation reaction mixtures of nucleic acid hydrolysates.

Derivative	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Average
	Ratios					
Pipsyluracil	0.384	0.392	0.395	0.388	0.396	0.391
Pipsylthymine	0.381	0.371	0.376	0.381		0.377

per cent of the I^{131} activity, high accuracy is favored when the $\bar{c}/\bar{s}$ ratio for the sample is about 0.3.

Analysis of Nucleic Acid Hydrolysates

Hydrolysis with Perchloric Acid—The quantitative aspects of the *perchloric acid method* of hydrolysis of nucleic acids (9) were reexamined by carrier and indicator procedures to follow the liberation of uracil and thymine from RNA and DNA, respectively. These analyses also demonstrated that the pipsylpyrimidines formed in reaction mixtures containing partial hydrolysis products of nucleic acids can be purified to constant specific activity. A slightly modified procedure for derivative formation was required for the $HClO_4$ hydrolysates. This procedure, described below, yielded satisfactory recovery analyses of uracil dissolved in $HClO_4$ (Table I).

An aliquot of unknown is transferred to a Folin sugar tube and the $HClO_4$ therein precipitated by adding an equivalent amount of tetramethylammonium hydroxide; the other reagents required for pipsylation are then added. The tube is stoppered and shaken for 1 hour on a high

frequency vibrator in order to disrupt the large tetramethylammonium perchlorate crystals in which small amounts of the bases are occluded. Upon acidification of the reaction mixture, the salt crystals dissolve. The analysis is then pursued by carrier or indicator techniques.

For the hydrolysis study, nucleic acid samples of approximately 100 mg. were accurately weighed into 10 ml. volumetric flasks, and 2 ml. of the desired concentration of $HClO_4$ were added. The flasks were stoppered and placed in a boiling water bath; at various intervals the flasks were removed, cooled in an ice bath, and diluted with water to 10 ml. The hydrolysates were then centrifuged to remove the caramel-like residue formed during the hydrolysis. Aliquots of 50 to 100 μl. (0.5 to 1 mg. of nucleic acid) were then taken for analysis. The results obtained (Table VII) are comparable to those reported by Marshak and Vogel (9). It is evident that DNA is more rapidly hydrolyzed than RNA and that treatment of these nucleic acids for 1 hour in 70 per cent $HClO_4$ effects complete release of uracil and thymine. The plateau region from 1 to 3 hours indicates that uracil and thymine are stable under the conditions of hydrolysis and that cytosine is not converted to uracil. The latter possibility was further checked by heating cytosine in 70 per cent $HClO_4$ at 100° for periods of 1 and 2½ hours and analyzing these solutions for uracil by the indicator technique. No significant conversion was detected (<0.01 per cent).

TABLE VII

Hydrolysis of Nucleic Acids in Perchloric Acid at 100°

The results are expressed as per cent recovery of uracil or thymine, the amount recovered in 70 per cent $HClO_4$ at 60 minutes being taken as 100 per cent.

Nucleic acid	$HClO_4$ concentration	Time of hydrolysis					
		10 min.	20 min.	40 min.	60 min.	120 min.	180 min.
		Per cent hydrolysis					
	per cent						
Yeast RNA	60	8.6	35	57	64		
" "	70	86	96	96	100	98	99
Calf thymus DNA	70	93	98	100	100	100	99

A comparison of some analyses obtained by both isotope derivative and conventional non-isotopic paper chromatographic methods (9, 10) on the same nucleic acid specimens hydrolyzed in 70 per cent $HClO_4$ (Table VIII) indicates good agreement between these two types of analysis.

Isotope derivative analyses have also been carried out on hydrolysates

of cell fractions in which compounds derived from constituents other than nucleic acids predominated. The pyrimidine content in some cases was less than 1 γ. It was nevertheless possible to purify the pipsylpyrimidine to constant specific activity, demonstrating that quantitative analyses for pyrimidines can be achieved without a preliminary isolation of the nucleic acid.

Hydrolysis with Formic Acid; Conversion of Cytosine to Uracil—It was found that cytosine is converted to uracil when nucleic acids are hydrolyzed in 92 per cent formic acid at 130° for long periods (20 to 400 hours). This observation suggested the possibility of determining cytosine by the isotope derivative method as additional uracil, since uracil *alone* can be

TABLE VIII

Comparison of Analyses Performed by Isotope Derivative and Conventional Non-Isotopic Paper Chromatographic Methods*

Nucleic acid	Isotope derivative	Paper chromatography
	Uracil	
	per cent	*per cent*
Yeast RNA	6.08	5.97†
	Thymine	
Calf thymus Na DNA	8.25	8.07‡

* Both methods were applied to hydrolysates of the same nucleic acid specimens.
† Value of Marshak and Vogel (9).
‡ Value obtained in this laboratory by the chromatographic system of Wyatt (10).

determined from $HClO_4$ hydrolysates. The rate of conversion of cytosine to uracil was then investigated. In 92 per cent formic acid, 0.3 ml. of formic acid per micromole of cytosine, at 140°, cytosine could be quantitatively converted to uracil in 120 hours. While cytosine has not been regularly determined in nucleic acids as additional uracil, this method has been used in a few instances for both RNA and DNA. Under the conditions for conversion of cytosine to uracil it is to be expected that 5-methylcytosine will be converted to thymine, and therefore it may be determined as additional thymine.

DISCUSSION

The theoretical basis for isotope derivative analysis (4) provides for a high degree of specificity and sensitivity which is required in many microanalytical problems in biology. Its theoretical and practical advantages

over isotope dilution and other types of analytical procedures have been demonstrated in its application to amino acid and peptide analysis (4, 5, 7, 11, 12).

In the isotope derivative method the accuracy of analysis is dependent on three fundamental factors: reproducible yield of isotopic derivative, isolation of pure isotopic derivative, and accurate isotope analysis (4). Stoichiometric yield is perhaps desirable, but the uncertainties surrounding the quantitative aspects of the reaction between pipsyl chloride and the pyrimidines need not be a cause for concern in view of the consistency of the recoveries. While the reason for the incompleteness of the yields is not known, certain factors may be eliminated; *i.e.*, formation of disubstituted derivatives, impure carriers or indicators, and impure pyrimidine standards. Constancy of specific activity has been taken as a sufficient criterion of the purity of the isolated isotopic derivative. This test, when carefully applied, is far more sensitive than the usual analytical or physical criteria of purity, and its adequacy has been demonstrated in previous work with the isotope derivative method (4, 5, 11, 12). Its application to the analysis of pyrimidines is supported by the data on the purification of isotopic derivatives, by the agreement between carrier and indicator analyses, and by the zero control analyses. It is evident from these considerations that the procedures described for the determination of pyrimidines fulfil the requirements stated above.

Previous attempts at accurate microanalysis of nucleic acid components in the presence of a wide variety of other compounds in hydrolysates of biological specimens have met with little success. Conventional paper chromatographic and microbiological methods have proved to be too insensitive or non-specific (9, 13), and, when isotope dilution was used (14), the determination was subject to gross error, owing to the very small dilution of the isotopic carrier (*cf.* (15)). On the other hand, the applicability of the isotope derivative method to these mixtures has been demonstrated by the attainment of constant specific activity of the pipsylpyrimidines isolated when such hydrolysates were analyzed.

SUMMARY

A scheme is presented for the analysis of microgram quantities of uracil and thymine by the isotope derivative method, with isotopic *p*-iodophenylsulfonyl (pipsyl) chloride as the analytical reagent. The method has been applied to the determination of uracil and thymine in nucleic acid hydrolysates. Data are also presented on the liberation of these pyrimidines from nucleic acids hydrolyzed in perchloric acid and on the conversion of cytosine to uracil in formic acid.

Complementary studies on the reaction between pipsyl chloride and pyrimidines and purines are reported.

BIBLIOGRAPHY

1. Warner, R. C., *Federation Proc.*, **8,** 264 (1949).
2. Schmidt, G., *Ann. Rev. Biochem.*, **19,** 149 (1950).
3. Jordan, D. O., *Ann. Rev. Biochem.*, **21,** 209 (1952).
4. Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, **71,** 249 (1949).
5. Keston, A. S., Udenfriend, S., and Levy, M., *J. Am. Chem. Soc.*, **72,** 748 (1950).
6. Shugar, D., and Fox, J. J., *Biochim. et biophys. acta*, **9,** 199 (1952).
7. Velick, S. F., and Udenfriend, S., *J. Biol. Chem.*, **190,** 721 (1951).
8. Levy, M., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **21,** 101 (1936).
9. Marshak, A., and Vogel, H. J., *J. Biol. Chem.*, **189,** 597 (1951).
10. Wyatt, G. R., *Biochem. J.*, **48,** 584 (1951).
11. Levy, M., and Slobodian, E., *J. Biol. Chem.*, **199,** 563 (1952).
12. Keston, A. S., and Udenfriend, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 92 (1949).
13. Elson, D., and Chargaff, E., *Experientia*, **8,** 143 (1952).
14. Marshak, A., and Marshak, C., *Exp. Cell Res.*, **5,** 288 (1953).
15. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133,** 737 (1940).

By

(*Fro*

The
this la
nucle
concer
series
guani
tained
others
colum
soluti
unity
preser

In
viewe
polyp
polya
acid
globir
prope
tionat
were
nucle
thetic
a mac
a basi
great
group

* T
Societ
Counc
and fr

† P

XUM

STUDIES ON NUCLEOPROTEINS

III. DEOXYRIBONUCLEIC ACID COMPLEXES WITH BASIC POLYELECTROLYTES AND THEIR FRACTIONAL EXTRACTION*

By PNINA SPITNIK,† RAKOMA LIPSHITZ, AND ERWIN CHARGAFF

(From the Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York)

(Received for publication, December 23, 1954)

The fractionation of deoxypentose nucleic acids, recently reported from this laboratory (1, 2), is essentially based on the fractional extraction of nucleohistone or artificial histone nucleates with salt solutions of increasing concentration in the presence of a chloroform phase. In this manner, a series of fractions of regularly graded composition (falling proportions of guanine and cytosine, rising proportions of adenine and thymine) is obtained. The fact that fractionation can be effected has been confirmed by others, either through the use of gradient elution from a histone-kieselguhr column (3) or in an arrangement in which the time of contact with the salt solution was varied rather than the concentration of the latter (4). The unity relationships previously stressed (5) and recently reviewed (6) were preserved in all fractions isolated in this laboratory.

In regard to its electrostatic properties, a deoxyribonucleic acid may be viewed, around pH 7, as a negatively charged polyelectrolyte, similar to a polyphosphoric acid, and histone as a basic polyelectrolyte not unlike a polyamine. In the instances cited above the fractionation of the nucleic acid was achieved by means of its reversible interaction with histone or globin. It appeared of interest to determine whether the electrostatic properties of the reacting compounds were the principal bases of the fractionation process or whether some structural peculiarities of the protein were also responsible. For this reason, the reaction of sodium deoxyribonucleate preparations from calf thymus with strongly electropositive synthetic polyelectrolytes was studied. One of the compounds, polylysine, is a macromolecular basic polypeptide and, on this account, not dissimilar to a basic protein, such as histone. Polyvinylamine, on the other hand, differs greatly from a protein, as it consists of a hydrocarbon chain to which amino groups are attached directly.

* This work has been supported by research grants from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, from the National Institutes of Health, United States Public Health Service, and from the Rockefeller Foundation.

† Present address, The Weizmann Institute of Science, Rehovoth, Israel.

XUM

The report on the study of the interaction between nucleic acid and these two polybases is here accompanied by an account of the fractionation of deoxyribonucleic acid by means of the complex with polylysine.

EXPERIMENTAL

Material

The specimen of sodium deoxyribonucleate was prepared from calf thymus by the procedure of Kay *et al.* (7). Polylysine hydrobromide (8) was a gift from the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel. For the sample of polyvinylamine hydrochloride we are greatly indebted to Dr. W. O. Kenyon, Eastman Kodak Company, Rochester, New York.

Analytical Procedures

Most of the methods used have been noted previously (1, 2, 9, 10). The amino nitrogen content of the polyelectrolyte preparations themselves was determined by the manometric method of Van Slyke; that of their solutions was calculated from the weight. The nucleic acid content of the complexes and solutions was computed from P determinations; in some cases measurement of the extinction at 260 mμ was used. Attempts to employ a modified biuret reaction (9) for the estimation of the quantity of polylysine remaining in solution after the precipitation of the nucleic acid complexes yielded values that, in contrast to those obtained with proteins, were only semiquantitative and which are not included here.

Preparation and Properties of Complexes

Regardless of whether, in the following experiments, equivalent amounts of polyelectrolyte amino N and nucleic acid P or an excess of one or the other reagent was employed, the order of addition, often not without influence on the quality of the resulting products (6, 11), was kept the same: the polybase solution was added, with stirring, to the solution of nucleic acid so that the latter remained in excess until the final concentration was reached.

Precipitation of Complexes from Water—The results of the experiments, in which polylysine (2 cc.) was mixed with nucleic acid (4 cc.) or polyvinylamine (2.5 cc.) with nucleic acid (5 cc.), all in distilled water, are summarized in Table I. White precipitates formed immediately, with the exception of the mixture described as Experiment 4 which remained turbid. The mixtures were shaken vigorously, stored at 3° for 48 hours, and centrifuged at 3000 × *g* for 5 minutes. Portions of the supernatant fluids, only faintly turbid except in Experiment 4, were analyzed and the results are presented in Table I. An additional precipitation could be produced by the addition

of salt to these fluids. In Experiments 1 to 4, the application of the biuret reaction (with polylysine as the standard) showed the proportion of polylysine remaining in solution to be dependent upon reagent concentration, as was that of the nucleic acid.

It will be seen that the percentage of nucleic acid precipitating from solution increased with increasing reagent concentration, though the relative proportions of the two partners were kept constant. Thus, with a ratio of amino nitrogen to phosphorus of 1, 66 per cent of the nucleic acid was precipitated in Experiment 1 and none in Experiment 4. These observa-

TABLE I

Precipitation from Distilled Water of Complexes between Basic Polyelectrolytes and Calf Thymus Deoxyribonucleic Acid

Polybase	Experiment No.	Concentration of reagents in mixture*			Nucleic acid P in supernatant fluid	Nucleic acid P in ppt.
		Polybase NH_2-N	Nucleic acid P	Ratio, $\frac{NH_2\text{-}N}{P}$		
		μmoles per cc.	*μmoles per cc.*		*μmoles per cc.*	*per cent of total P*
Polylysine	1	3.91	3.76	1.04	1.27	66
	2	1.96	1.87	1.05	1.07	43
	3	0.98	0.98	1.00	0.84	14
	4	0.49	0.49	1.00	0.50	0
Polyvinylamine	5	3.92	3.95	0.99	0.23	94
	6	1.96	1.97	0.99	0.23	88
	7	0.98	0.99	0.99	0.19	81
	8	0.49	0.49	1.00	0.19	61

* The total volume was 6 cc. in Experiments 1 to 4, 7.5 cc. in Experiments 5 to 8.

tions are in accord with those made by Alexander (12) with mixtures of nucleic acid and protamine.

Complexes Soluble in Water—As the concentration of the interacting polyelectrolytes is lowered, a point is reached at which no precipitate is formed. That there still occurs an interaction between the nucleic acid and the polybase in these clear aqueous solutions is evidenced by the experiments summarized in Table II. When very dilute, aqueous solutions of sodium deoxyribonucleate and of basic polyelectrolytes, in the indicated concentrations, were mixed (total volume 50 cc.), clear solutions resulted whose extinction at 260 mμ was determined. During concentration in a vacuum at 35° in a "flash evaporator" the solutions remained perfectly clear, depositing a thin film on the walls of the flask after the water was removed. Each residue was extracted with 50 cc. of water and the extinction in the ultraviolet of the solution was again determined.

XUM

As can be seen in Table II, the solubility of the films, or at any rate of their nucleic acid portions, was a function of the ratio of polybase amino groups to nucleic acid phosphorus present initially in the solutions from which they had been isolated. With a ratio of 1 the films were insoluble (Experiments 10 and 17). An excess of either type of ionic group, but especially of phosphate, increased the solubility.

TABLE II

Formation in Distilled Water of Soluble Complexes between Basic Polyelectrolytes and Calf Thymus Deoxyribonucleic Acid

Polybase	Experiment No.	Concentration of reagents in mixture*			Proportion of nucleic acid soluble after evaporation of mixture†
		Polybase NH_2-N	Nucleic acid P	Ratio, $\frac{NH_2\text{-}N}{P}$	
		μmole per cc.	*μmole per cc.*		*per cent of extinction*
Polylysine	9	0.029	0.057	0.5	81
	10	0.029	0.029	1.0	0
	11	0.043	0.029	1.5	0
	12	0.086	0.029	3.0	0
	13	0.171	0.029	5.9	9
	14	0.343	0.029	11.8	13.5
Polyvinylamine	15	0.029	0.113	0.25	100
	16	0.029	0.057	0.5	28
	17	0.029	0.028	1.0	0
	18	0.114	0.028	4.1	8
	19	0.229	0.028	8.2	32
	20	0.343	0.028	12.2	87

* The total volume was 50 cc. in all experiments.

† The film, deposited on evaporation of the mixture, was treated with 50 cc. of water and the extinction at 260 mμ of the resulting solution was compared with that of the original clear mixture of reagents.

Precipitation of Complexes from Salt Solution—The experiments on the formation of complexes between polylysine and deoxyribonucleic acid, described in Table I as Experiments 1 to 4, were repeated with aqueous 0.5 M NaCl, instead of distilled water, as the solvent. When the reagents were mixed in equimolar concentration over a range of 4.0 to 0.5 μmole per cc. of amino nitrogen and nucleic acid phosphorus, the latter was found entirely in the precipitates. In contrast to the experiments in distilled water, less than 0.05 per cent of the nucleic acid remained in the supernatant fluids which showed no turbidity.

Another method of studying the precipitation of nucleic acid-polybase complexes from salt solution is exemplified in Tables III and IV. Mixtures of nucleic acid and polylysine in 2 M aqueous sodium chloride were

prepared to form solutions of twice the reagent concentrations shown in Table III. After being stored in the cold for 24 hours, portions of each solution were diluted with an equal volume of NaCl solution of appropriate strength to form the mixtures listed in Table III. At this point, precipitation occurred in several instances. The mixtures were shaken vigorously and kept in the cold for 48 hours, and the supernatant fluids (centrifugation at 3000 × g for 10 minutes) were analyzed for P. Biuret determinations showed the amounts of polylysine remaining in solution to be roughly parallel to that of phosphorus.

In contrast to the complexes with polylysine those formed with polyvinylamine were not dissolved by 2 M NaCl. Precipitates produced in

TABLE III

Precipitation of Complexes between Polylysine and Calf Thymus Deoxyribonucleic Acid Soluble in 2 M NaCl

Experiment No.	Concentration of reagents in mixture*			Nucleic acid P in ppt. produced by adjustment to lower salt concentration			
	Polylysine NH_2-N	Nucleic acid P	Ratio, $\frac{NH_2\text{-}N}{P}$	1.0 M	1.25 M	1.5 M	1.75 M
	μmoles per cc.	*μmoles per cc.*		*per cent of total P*	*per cent of total P*	*per cent of total P*	*per cent of total P*
21	1.98	1.61	1.23	94	64	0	0
22	0.99	0.84	1.18	88	4	0	0
23	0.50	0.42	1.19	71	2	0	0
24	0.25	0.21	1.19	43	0	0	0

* After adjustment to indicated salt concentration; total volume, 6 cc.

aqueous solutions of nucleic acid and polyvinylamine remained, in fact, insoluble at an NaCl concentration as high as 4 M. Magnesium salts, either the chloride or the acetate, effected, however, the partial solubilization of such products at a higher than M salt concentration. The ease with which these complexes went into solution appeared to parallel the reagent concentration at which they were formed. But solubilization was far from complete under the conditions of the present study; for this reason, no polyvinylamine complexes were employed in the fractionation studies described later in this paper.

A series of experiments with polyvinylamine similar to those with polylysine described in Table III, but employing magnesium acetate, is shown in Table IV. The experimental conditions were the same. The mixtures in 2 M magnesium acetate were very viscous; the first appearance of a precipitate on adjustment to a lower salt concentration was accompanied by a sharp drop in viscosity.

These dilution experiments show that two factors are of importance for the solubility of a given polybase-nucleic acid complex: (*a*) the proportion of insoluble product increases with decreasing salt concentration; (*b*) below a certain critical salt concentration, the solubility of the complex is inversely

TABLE IV

Precipitation of Complexes between Polyvinylamine and Calf Thymus Deoxyribonucleic Acid Soluble in 2 M Magnesium Acetate

Experiment No.	Concentration of reagents in mixture*			Nucleic acid P in ppt. produced by adjustment to lower salt concentration			
	Polyvinylamine NH_2-N	Nucleic acid P	Ratio, $\frac{NH_2\text{-}N}{P}$	1.0 M	1.25 M	1.42 M	1.66 M
	μmoles per cc.	*μmoles per cc.*		*per cent of total P*	*per cent of total P*	*per cent of total P*	*per cent of total P*
25	1.95	1.81	1.08	96	85	4	0
26	0.98	0.90	1.09	93	64	5	0
27	0.45	0.42	1.07	92	60	4	0
28	0.24	0.23	1.04	50	4	5	0

* After adjustment to indicated salt concentration; total volume, 6 cc.

TABLE V

Fractional Extraction of Complexes between Polylysine and Calf Thymus Deoxyribonucleic Acid; Experimental Conditions and Phosphorus Distribution

Experiment No.	Nucleic acid P in mixture*	Total volume of mixture	Nucleic acid P in ppt.	Per cent of nucleic acid P extracted at indicated molarity of NaCl								Nucleic acid P extracted
				1.0	1.15	1.25	1.5	1.75	2.0	2.5	4.0	
	μmoles per cc.	*cc.*	*per cent of total P*									*per cent of P in complex*
29	2.48	138	100	1.5		54.3	35.3	1.0				92.1
30	1.71	233	94	0.2		40.8	43.2	9.5				93.7
31	1.50	302	99		23.9	21.3	22.1	10.3		2.4		80.0
32	1.50	294	100		33.9	19.9	18.6	1.9				74.3
33	0.84	616										
34	0.75	290	90	0.7	16.4	10.5	25.9	19.6	9.6		3.1	85.8

* The molar ratio of polylysine amino N to nucleic acid P was 1.0 in all experiments.

related to the concentration of reagents at which it was formed initially (compare, for instance, Experiments 21 and 25 with 24 and 28).

Fractionation of Sodium Deoxyribonucleate by Fractional Extraction of Polylysine Complexes

The experiments summarized in Tables V and VI were carried out with complexes of polylysine and sodium deoxyribonucleate prepared in 0.5 M

NaCl solution. These products are essentially insoluble in M NaCl. The treatment with salt solutions of increasing strength brings about the grad-

TABLE VI

Fractional Extraction of Complexes between Polylysine and Calf Thymus Deoxyribonucleic Acid; Composition of Fractions*

Experiment No.	Fraction No.	NaCl	Moles per 100 gm. atoms P†					Molar ratios			
			Total recovery	A	G	C	T	$\frac{A+T}{G+C}$	$\frac{A}{T}$	$\frac{G}{C}$	$\frac{Pu}{Py}$
		M									
Total DNA‡				29.0	21.2	21.2	28.7	1.36	1.01	1.01	1.01
29	I	1.25	92.2	26.8	21.8	23.1	28.2	1.23	0.95	0.94	0.95
	II	1.5	101.2	29.4	19.7	20.6	30.3	1.48	0.97	0.96	0.96
30	I	1.25	103.7	27.5	23.4	22.6	26.5	1.17	1.04	1.03	1.04
	II	1.5	96.3	30.6	20.7	19.2	29.5	1.51	1.04	1.08	1.05
	III	1.75	105.2	29.7	19.5	20.3	30.5	1.51	0.97	0.96	0.97
31	I	1.15	98.2	28.0	22.4	21.7	28.0	1.27	1.00	1.03	1.01
	II	1.25	95.6	31.1	19.1	19.3	30.5	1.60	1.02	0.99	1.01
	III	1.5	96.5	28.8	19.8	21.3	30.0	1.43	0.96	0.93	0.95
	IV	1.75	106.5	29.3	20.7	21.3	28.7	1.38	1.02	0.97	1.00
32	I	1.15	96.0	29.3	21.6	21.3	27.9	1.33	1.05	1.01	1.03
	II§	1.15	91.2	29.2	21.2	20.5	29.0	1.40	1.01	1.03	1.02
	III	1.25	100.7	29.4	20.4	20.8	29.4	1.43	1.00	0.98	0.99
	IV	1.5	94.0	29.0	21.0	20.1	29.9	1.43	0.97	1.04	1.00
33	I	1.25	97.0	26.5	23.4	22.9	27.1	1.16	0.98	1.02	1.00
	II	1.5	98.9	31.0	19.1	19.0	30.9	1.62	1.00	1.00	1.00
	III	1.75	92.5	30.8	18.9	18.9	31.4	1.65	0.98	1.00	0.99
	IV	2.5	103.7	30.7	19.9	19.8	29.6	1.52	1.04	1.00	1.02
	V	Saturated	103.4	29.2	19.3	20.0	31.5	1.54	0.93	0.97	0.94

* Compare Table V for experimental conditions.

† The total average recovery of moles of nitrogenous constituents per 100 gm. atoms of P is indicated for each fraction. The mean proportions of each constituent have been corrected to a 100 per cent recovery. The following abbreviations are used: A, adenine; G, guanine; C, cytosine + 5-methylcytosine; T, thymine; Pu, purines; Py, pyrimidines.

‡ Average composition of ox deoxyribonucleic acid taken from a previous paper (10).

§ Two consecutive extracts at the same NaCl concentration were examined in this instance.

ual solubilization of the complexes, which had been prepared and collected as described before. The precipitates obtained in Experiments 29, 30, and 34 (Table V) were each washed twice with 0.5 M NaCl and intimately suspended in 50 cc. of M NaCl. The mixtures were kept overnight at room temperature and then centrifuged at 4000 × *g*. The extraction with fresh

portions of the same molarity was repeated until the supernatant fluids contained little additional P. These operations were carried out in succession with the salt solutions listed in Table V and the extracts collected at each molarity were combined.

The complexes prepared in Experiments 31 to 33 were not extracted exhaustively, but were, instead, treated twice at each salt concentration with 50 cc. portions in a high speed mixer for 1 minute. The immediate centrifugation of the mixture at 20,000 × *g* for 10 minutes was followed by the next extraction of the undissolved residue. This procedure is more rapid and effective, but wasteful. The extracts afforded at each stage were combined, except in Experiment 32 in which the two nucleic acid fractions isolated in 1.15 M NaCl were analyzed separately. Experiment 33 was performed for the sole purpose of securing nucleic acid fractions and was not followed in a quantitative manner. All extracts contained, in addition to nucleic acid, polylysine as shown by the biuret test.

The portions that had gone into solution in the respective NaCl solutions were recovered either by successive dialysis against 2.5 M NaCl, running tap water and distilled water, and lyophilization, or by precipitation with 2 volumes of ethanol from the solution that had been adjusted previously to a 2.6 or 4 M NaCl concentration. In the latter case, the precipitates were washed with 70 and 95 per cent ethanol and with acetone and dried in air. In contrast to the behavior of nucleohistone (2, 9), the precipitation failed to eliminate the basic polyelectrolyte.

The nucleic acid fractions secured by the fractional extraction were analyzed in the usual manner and the results are presented in Table VI. With the exception of Experiment 32, the data recorded here show that fractionation had been achieved.

DISCUSSION

In regard to the interaction between nucleic acids and proteins reference may be made to a brief discussion recently given by one of us (6) which will not be repeated here. There can be little doubt that the multiple possibilities of cross-linking taking place between the partners may give rise to many different products which will differ in their properties (2, 6, 11). For this reason, it should be emphasized that the order of mixing was kept uniform throughout all experiments reported here, the basic polyelectrolyte always being added to the solution of nucleic acid, and that, with the exception of the studies summarized in Table II, equimolar quantities of the reagents were employed.

The precipitation of complexes from distilled water, illustrated in Table I, actually took place in solutions that were 0.0005 to 0.004 M with respect to NaBr or NaCl, since salts of the two partners were employed. The

solubility of complexes thus prepared appears to depend mainly upon the initial concentration of reagents. The inverse relationship between initial reagent concentration and solubility is paralleled by the results of the experiments on the precipitation of complexes by the dilution of salt solutions of high ionic strength (Tables III and IV).

At very low reagent concentrations, compounds were formed with both basic polyelectrolytes that were soluble in distilled water and did not precipitate during the evaporation of the solvent. The ability of the residual films, once they had been isolated, to redissolve in water was, however, a function of the relative proportions of the reagents used for the preparation (Table II). The production of soluble complexes between nucleic acid and polylysine or polyvinylamine recalls similar observations by Alexander (12) on the formation of soluble protamine nucleates. It may be assumed that under conditions unfavorable for cross-linking, *i.e.* when the macromolecules are too far apart, linear complexes are formed in which 1 or more molecules of the polybase are aligned lengthwise on a single nucleic acid molecule. Concerning the solubility properties of films produced from such solutions, reference may also be made to studies of Ambrose and Butler (13) on films of nucleohistone.

As was to be expected, the properties of the complexes of nucleic acid with histone, polylysine, and polyvinylamine showed many similarities. It may be assumed that when a nucleic acid and a polybase are brought into contact in a solution of sufficient concentration, three-dimensional, cross-linked networks are formed by means of salt bridges. As the complex is formed, the oppositely charged macromolecules neutralize each other, reducing the net charge and decreasing the solubility. In measure with a rise in salt concentration, first a salting-out effect is noticeable, but, as the ionic strength increases further, the salt penetrates the complex and, by screening off the combining groups, brings about the gradual dissociation of the complex. (Compare, for instance, the findings of Crampton *et al.* (9) on nucleohistone.)

Complexes of nucleic acid with histone begin to go into solution at NaCl concentrations above 0.5 M, those with polylysine above M, and complexes with polyvinylamine require M or stronger solutions of Mg^{++} ion. Little is known about the electrostatic interaction of polyelectrolytes; but the charge density of the polyelectrolyte appears to be an important factor (14–17). Estimates of the arginine and lysine contents of histone vary over a rather wide range (18); even the highest figure of a total of about 43 per cent (19, 20) indicates that the cationic density of this protein is lower than that of polylysine, which is composed entirely of lysine residues. On the other hand, while the amino groups in the latter polyelectrolyte are separated from the polypeptide backbone by lateral chains composed of

four methylene groups, they are attached directly to the hydrocarbon chain of polyvinylamine, which must have the highest charge density of the compounds under comparison. The observation that polyvinylamine complexes were less soluble than polylysine complexes, whereas the compounds with histone were more soluble and more easily dissociated than the latter, is in agreement with this order of charge densities.

The experiments reported in Tables I and III to V, in which the complexes were formed at identical ionic strengths and reagent ratios, demonstrate a direct relationship between the initial concentration of reagents and both the quantity of complex precipitating and the ease of its subsequent solubilization by higher salt concentrations.

The experiments on the fractional extraction of nucleic acid from the compounds with polylysine are not without some interest, as they show that a synthetic polybase can take the place of a protein in this separation. The data assembled in Table VI suggest that in four out of five instances a fractionation with respect to base composition has been achieved, as shown by the ratio of adenine + thymine to guanine + cytosine. No reason can be given for the failure in Experiment 32. The trend resembled that observed in our previous studies on fractionation by means of histone (1, 2); the guanine and cytosine contents diminished and the adenine and thymine proportions rose, in two succeeding fractions, though in the present experiments fewer preparations of discrete composition appear to have been separated. It cannot yet be stated whether much significance should be attributed to the observation that in the present experiments the last fractions collected (Experiment 31, Fractions III and IV; Experiment 33, Fractions IV and V) showed a somewhat lower ratio of adenine + thymine to guanine + cytosine than did the preceding ones. The unity relationships between adenine and thymine and between guanine and cytosine were maintained, and so was the equality of the proportions of purines and pyrimidines (5). The principles underlying the separation procedure have been considered previously (1–3, 6).

SUMMARY

The properties of complexes between calf thymus deoxyribonucleic acid and the synthetic basic polyelectrolytes polylysine and polyvinylamine have been studied. The solubility of complexes formed at identical ionic strength and reagent ratios decreased with increasing reagent concentration. On the other hand, the higher the initial concentration of nucleic acid and polybase, the greater the ease with which the complex could be brought into solution at a high ionic strength. At extremely low concentrations, complexes between nucleic acid and polylysine or polyvinylamine were produced that were soluble in water.

As had been observed previously during the study of the fractional dissociation of nucleohistone, the fractional extraction of complexes with polylysine by salt solutions of increasing strength resulted in the separation of differently composed nucleic acid fractions.

BIBLIOGRAPHY

1. Chargaff, E., Crampton, C. F., and Lipshitz, R., *Nature*, **172,** 289 (1953).
2. Crampton, C. F., Lipshitz, R., and Chargaff, E., *J. Biol. Chem.*, **211,** 125 (1954).
3. Brown, G. L., and Watson, M., *Nature*, **172,** 339 (1953).
4. Lucy, J. A., and Butler, J. A. V., *Nature*, **174,** 32 (1954).
5. Chargaff, E., *Experientia*, **6,** 201 (1950); *J. Cell. and Comp. Physiol.*, **38,** suppl. 1, 41 (1951); *Federation Proc.*, **10,** 654 (1951).
6. Chargaff, E., in Chargaff, E., and Davidson, J. N., The nucleic acids, New York, **1,** 307 (1955).
7. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., *J. Am. Chem. Soc.*, **74,** 1724 (1952).
8. Katchalski, E., Grossfeld, I., and Frankel, M., *J. Am. Chem. Soc.*, **70,** 2094 (1948).
9. Crampton, C. F., Lipshitz, R., and Chargaff, E., *J. Biol. Chem.*, **206,** 499 (1954).
10. Chargaff, E., and Lipshitz, R., *J. Am. Chem. Soc.*, **75,** 3658 (1953).
11. Chargaff, E., *Symposia Soc. Exp. Biol.*, in press.
12. Alexander, P., *Biochim. et biophys. acta*, **10,** 595 (1953).
13. Ambrose, E. J., and Butler, J. A. V., *Discussions Faraday Soc.*, **13,** 261 (1953).
14. Burger, W. C., and Stahmann, M. A., *J. Biol. Chem.*, **193,** 13 (1951).
15. Morawetz, H., and Hughes, W. L., Jr., *J. Phys. Chem.*, **56,** 64 (1952).
16. Oncley, J. L., Ellenbogen, E., Gitlin, D., and Gurd, F. R. N., *J. Phys. Chem.*, **56,** 85 (1952).
17. Rice, R. V., Stahmann, M. A., and Alberty, R. A., *J. Biol. Chem.*, **209,** 105 (1954).
18. Tristram, G. R., in Neurath, H., and Bailey, K., The proteins, New York, **1,** 181 (1953).
19. Brunish, R., Fairley, D., and Luck, J. M., *Nature*, **168,** 82 (1951).
20. Luck, J. M., Kupke, D. W., Rhein, A., and Hurd, M., *J. Biol. Chem.*, **205,** 235 (1953).

THE INHIBITION OF TRYPSIN

III. INFLUENCE OF UREA*

By T. VISWANATHA† and IRVIN E. LIENER

(From the Department of Agricultural Biochemistry, University of Minnesota, St. Paul, Minnesota)

(Received for publication, January 28, 1955)

It is well recognized that many proteolytic enzymes, including trypsin (1, 2), papain (3), asclepain (4), and certain bacterial proteinases (5), attack denatured proteins much more readily than proteins in their native form. Urea is frequently employed as the denaturant because of its dispersive action on the otherwise insoluble denatured protein. Proteolytic activity with denatured proteins is generally measured by adding the enzyme directly to the protein in the presence of high concentrations of urea. Such a procedure can be justified only if (*a*) the enzyme is resistant to urea denaturation, or (*b*) the enzyme, although susceptible to denaturation by urea, is protected by the substrate.

Papain is an example of (*a*), since it has been shown to be remarkably stable to concentrations of urea as high as 9 M even in the absence of substrate (6). Chymotrypsins α, β, and γ, on the other hand, seem to exemplify condition (*b*), since these enzymes are completely inactivated by 4 M urea in the absence of substrate, whereas they readily attack hemoglobin in the presence of 5 M urea (7). Although pepsin does not require denatured protein as a substrate, neither is it inactivated by prolonged exposure to 4 M urea (8).

In view of the fact that the activity of trypsin is frequently measured on denatured hemoglobin in the presence of a high concentration of urea (9), it is indeed surprising that studies relating to the effect of urea on trypsin in the absence of substrate have not been reported.[1] The present study was designed to supply information on this point.

EXPERIMENTAL

The trypsin used was a twice crystallized product containing 50 per cent $MgSO_4$ purchased from the Worthington Biochemical Corporation, Free-

* Paper No. 3314, Scientific Journal Series, Minnesota Agricultural Experiment Station, University of Minnesota.

† Partial support from the J. N. Tata Endowment Fund is gratefully acknowledged.

[1] To the authors' knowledge the only exception to this statement is the report by Schales (10) that 0.10 M urea did not inhibit trypsin. This concentration of urea, however, is far below the level ordinarily required for protein denaturation.

XUM

hold, New Jersey. The enzyme was freed of salt by extensive dialysis against 0.001 N HCl, followed by lyophilization.

Requisite amounts of urea (Baker's reagent grade) were dissolved in about 60 ml. of 0.05 M borate buffer, pH 7.6. The pH was readjusted to 7.6 and finally diluted to 100 ml. with buffer.

A stock solution of trypsin was prepared prior to each run in the appropriate buffered urea solution, and the concentration of trypsin protein (mg. per ml.) was calculated from the absorbance at 280 mμ by a conversion factor of 0.585 (11). The desired trypsin concentration was obtained by further dilution with the appropriate buffered urea solution.

Enzyme-urea solutions were placed in a water bath at 37°, and the activity was determined periodically by transferring 1 ml. aliquots to a mixture of 1 ml. of 0.05 M borate buffer, pH 7.6, and 3 ml. of 2 per cent casein (Hammarsten quality) prepared in the same buffer. Tryptic activity was evaluated by measuring the absorbance of the deproteinized filtrate at 280 mμ according to the technique of Kunitz (12). Appropriate blanks were also included in which trypsin-urea mixtures were added to the substrate after precipitation of the latter with trichloroacetic acid (TCA). "Initial activity" (activity at zero time) as used hereafter refers to the proteolytic activity observed in exactly 10 minutes after dissolving the enzyme in the appropriate buffered urea solution.[2] All subsequent activity measurements have been calculated as the per cent of the initial activity.

Results

Effect of Urea Concentration on Trypsin Activity—The data pertaining to the influence of various concentrations of urea, ranging from 0 to 8.3 M, are shown in Fig. 1.[3] Even in the absence of urea, trypsin underwent a certain loss in activity, presumably as the result of autolysis which is favored by the conditions of pH and temperature employed here (11). Urea concentrations up to 2.5 M did not increase the rate at which trypsin activity was lost. In fact, this loss was somewhat retarded by urea concentrations of 0.8 and 1.7 M. Concentrations of urea from 3.3 to 5.0 M accelerated the rate of inactivation, whereas 5.8 M urea reversed this trend until complete stability was obtained at 6.7 and 8.3 M urea.

[2] The initial activity varied with the concentration of urea between 5.0 M and 6.7 M and reflected the loss in enzymatic activity during the 10 minutes required for preparation of the solutions.

[3] It has not been possible to express the rate of inactivation in terms of characteristic rate constants, since the order of the reaction varied with the concentration of urea. Only below 2.5 M urea did the loss in activity follow a second order reaction, which agrees with what has been reported for the autolysis of trypsin in the absence of urea (13, 14).

Appearance of TCA-Soluble Products—Since the losses in activity shown by the curves in Fig. 1 were dependent on time, a process of autolysis was indicated, in which case it should be possible to demonstrate the formation of split-products soluble in TCA. Solutions of trypsin in various concentrations of urea buffered at pH 7.6 were allowed to stand at 37°, and TCA was added at various intervals. The absorbance of the TCA filtrates was measured at 280 mμ. Trypsin, in the absence of urea, displayed the anticipated appearance of TCA-soluble products (Fig. 2); little change was effected by exposure to 0.8 M urea. Urea concentrations of 3.3 and 5.0 M

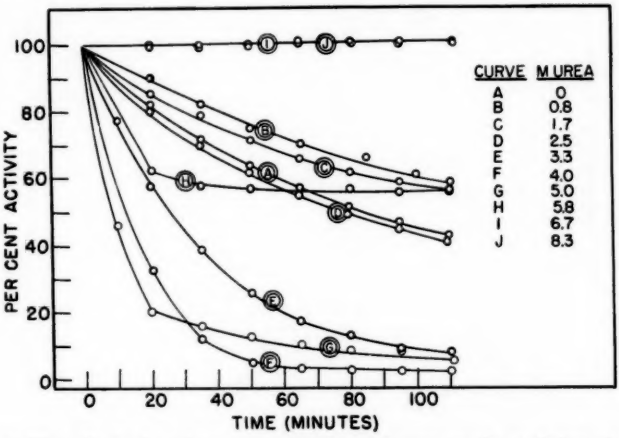


FIG. 1. The effect of various concentrations of urea on the activity of trypsin. Solutions containing 100 γ of trypsin per ml. and various concentrations of urea buffered at pH 7.6 were allowed to stand at 37°. At various intervals the proteolytic activity of 1 ml. aliquots was determined as described in the text.

appreciably increased the rate at which trypsin was hydrolyzed. At higher concentrations of urea, 6.7 and 8.3 M, the TCA-soluble products remained at a low constant value. The failure to obtain an initial absorbance value of zero at the higher levels of urea could be due in part to incomplete precipitation of intact trypsin by TCA. However, the fact that the absorbance of the 8.3 M urea system was less than that of the 6.7 M urea system indicates that this may not be the sole explanation.

Effect of Calcium—Since calcium ions have been shown to retard the autolysis of trypsin (15, 16), the effect of Ca^{++} on the action of urea on trypsin was investigated. Trypsin activity was measured in the presence of various concentrations of urea as previously described, with the exception that sufficient $CaCl_2$ was added to the various enzyme-urea systems to give a final concentration of 0.01 M. The results of this study are presented in

Fig. 3. In contrast to the losses in activity previously recorded in Fig. 1, trypsin exposed to levels of urea ranging from 0 to 3.3 M now retained most of its activity. Although 5.0 M urea caused some loss in activity in spite of added Ca^{++}, this loss was considerably less than that which had been observed in the absence of Ca^{++}. Somewhat unexpected was the rapid inactivation caused by 6.7 M urea in the presence of Ca^{++}, although this

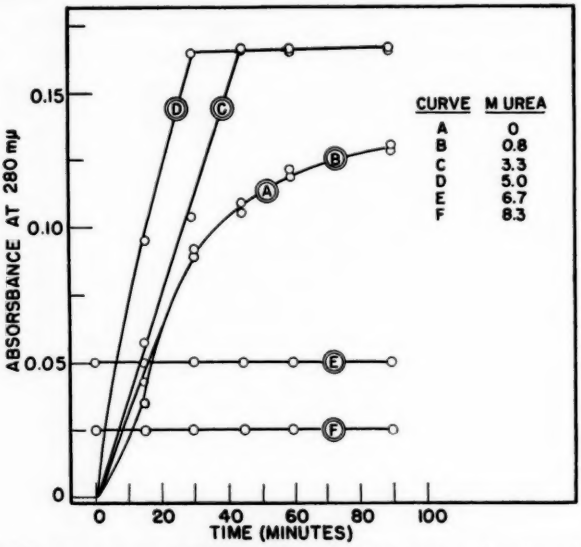


FIG. 2. The appearance of TCA-soluble products from trypsin exposed to various levels of urea. Solutions containing 400 γ of trypsin per ml. and various concentrations of urea buffered at pH 7.6 were allowed to stand at 37°. At various intervals 3 ml. aliquots were deproteinized with 7 ml. of 5 per cent TCA, and the absorbance of the filtrate was measured at 280 mμ.

same level of urea without added Ca^{++} had produced a stable system. The stability of trypsin in 8.3 M urea was still evident in the presence of Ca^{++}.

Effect of Sodium Decylbenzene Sulfonate (DBS)—It had been previously reported (17) that certain critical concentrations of detergents, particularly DBS, prevented the autolysis of trypsin. A study was therefore made of urea inactivation in the presence of a concentration of DBS known to stabilize trypsin. The marked instability of trypsin in 5.0 M urea was effectively prevented in the presence of 10^{-3} M DBS (Fig. 4). In agreement with an earlier report (17) the DBS-trypsin complex was only 70 per cent as active as the original enzyme, but, in contrast to the latter, was very stable in the presence of 5.0 M urea.

Effect of pH—Since the autolysis of trypsin is known to be considerably retarded under acid conditions (18), the effect of urea on trypsin activity was studied at pH 4. The experimental details were identical with those conducted at pH 7.6 except that 0.1 M acetate buffer replaced the borate

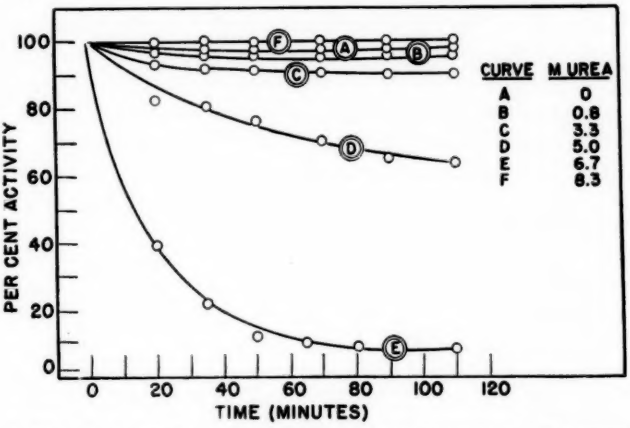


FIG. 3. The effect of Ca^{++} on the urea inactivation of trypsin. The experimental details were the same as for Fig. 1, except that all trypsin-urea systems contained 0.01 M $CaCl_2$.

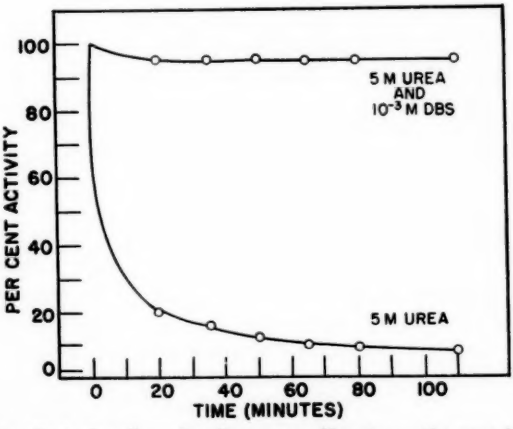


FIG. 4. The effect of sodium decylbenzene sulfonate on the urea inactivation of trypsin. Solutions containing 100 γ of trypsin per ml. in 5 M urea, pH 7.6, in the presence or absence of 10^{-3} M DBS, were allowed to stand at 37°. At various periods of time, 1 ml. aliquots were removed for determination of activity.

buffer in order to maintain a pH of 4. Under these conditions, no significant loss in activity was noted over a period of 2 hours at any concentration of urea ranging from 0 to 8.3 M. A repetition of this experiment at pH 6 with 0.05 M phosphate buffer yielded results which were qualitatively similar to those obtained at pH 7.6 (Fig. 1), although inactivation at urea concentrations of 3 to 5 M was not as pronounced as in the latter instance.

Effect of Dilution—In the experiments described thus far the activity was determined by adding the enzyme-urea solution directly to the substrate. It was noted, however, that if trypsin solutions which had been stabilized with 6.7 or 8.3 M urea were diluted with an equal volume of buffer

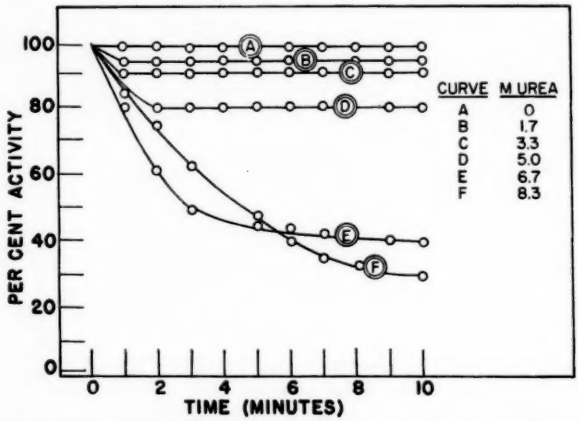


FIG. 5. Effect of dilution of trypsin-urea systems. 1 ml. portions of solutions containing 100 γ of trypsin per ml. in various concentrations of urea, pH 7.6, were diluted with 1 ml. of 0.05 M borate buffer, pH 7.6. At various intervals 3 ml. of the casein substrate were added, and the activity was determined as described in the text.

and allowed to stand before the substrate was added, activity was rapidly lost. This effect is illustrated in Fig. 5. Although dilution caused some inactivation at lower concentrations of urea, this usually occurred within the 1st minute and remained essentially unchanged during the remainder of the 10 minute period. The rapid loss in activity which followed dilution of initially stabilized trypsin-urea systems did not appear to involve autolysis, which is a much slower process. This view was confirmed by the finding that TCA filtrates of diluted trypsin-urea systems showed no appreciable increase in absorbance at 280 mμ.

DISCUSSION

The loss in trypsin activity produced at pH 7.6 by concentrations of urea ranging from 3.3 to 5.0 M and the appearance of TCA-soluble products

under the same conditions appear to be concordant manifestations of an autolytic reaction. According to Kunitz and Northrop (13) the autolysis of trypsin proceeds according to the reactions

$$\text{Ta} \rightleftharpoons \text{Ti} \tag{1}$$

$$\text{Ta} + \text{Ti} \rightleftharpoons \text{TaTi} \rightarrow \text{Ta} + \text{split-products} \tag{2}$$

where Ta is the native, active protein and Ti is a reversibly denatured, inactive protein. It becomes pertinent, therefore, to ascertain in what manner urea participates in this scheme to accelerate the autolytic process.

The fact that trypsin could not be inactivated by urea under acid conditions brings to mind the suggestion by Neurath *et al.* (19) that the denaturation of trypsin may involve a dissociation of the free carboxyl groups of the trypsin molecule. Hence at pH 4, at which complete protection against urea was noted, the dissociation of the free carboxyls would be repressed (20) and the existence of Ta would be favored. The failure of urea to denature trypsin at pH 4 could then be explained by assuming that Ta is less susceptible to the action of urea than Ti. At pH 7.6, however, urea may interact with trypsin, Ti being the most likely target of attack, and the nature of this interaction may be similar to that of ovalbumin and urea (21) in which a series of complexes is formed, depending on the concentration of urea. In the case of 3.3 to 5.0 M urea these complexes are probably unstable and unfold at a characteristic rate to give denatured protein. The latter would then be subjected to degradation in a reaction similar to Equation 2, leading to an irreversible loss in activity and the appearance of TCA-soluble products. Urea would be expected to enhance the rate of autolysis by providing an increased level of substrate in the form of denatured protein.

The above considerations likewise provide an explanation for the protective influence of substrate on the urea inactivation of trypsin when enzymatic activity is measured on denatured hemoglobin. In the method of Anson and Mirsky (9) the final concentration of urea after mixing the enzyme solution with the urea-containing substrate is about 5.5 M, a concentration which would be expected to cause appreciable inactivation in the absence of substrate.[4] According to these authors (9), the conditions of their test favor the conversion of Ti to Ta, a situation which would preclude appreciable urea inactivation if the above assumptions are correct.

The marked stability afforded to trypsin by higher concentrations of urea, 6.7 and 8.3 M, bears a striking resemblance to the active, stable trypsin-detergent complex previously noted with certain concentrations of DBS (17). It is not clear, however, why such a complex should fail to unfold

[4] In the presence of substrate (casein) 4 M urea decreased trypsin activity by 15 to 20 per cent compared with a non-urea control. This should be compared with the almost complete loss in activity in the absence of substrate (Curve F, Fig. 1).

to denatured protein. Upon dilution a rapid loss in activity does ensue which may be due to the loss of loosely bound urea responsible for stabilization. Since, at very high concentrations of urea, all of the enzyme would be in the form of an active urea complex, the denatured protein formed upon dilution would not be subjected to further enzymatic attack. This would account for the much more rapid loss in activity than that which characterizes autolysis and for the absence of products soluble in TCA.

At lower concentrations of urea, 3.3 to 5.0 M, the relatively small losses in activity occurring within the 1st minute following dilution may be a reflection of the amount of protein which has been denatured by an initial exposure to urea. The activity appears to be constant thereafter, since the final concentration of urea has been reduced to a level at which further denaturation would not be expected (<2.5 M).

The partial protection of trypsin by Ca^{++} against concentrations of urea which ordinarily inactivate it may be related to the several lines of evidence which indicate that Ca^{++} forms an enzymatically active complex with trypsin (16, 20, 22). This complex may require more urea to produce an unstable intermediate, and, by the same token, more urea would be required to produce the active, stable complex. This would explain why 6.7 M urea, which would ordinarily produce a stable trypsin system, does in fact rapidly inactivate the enzyme in the presence of Ca^{++}. The latter effect corresponds to what is observed with lower concentrations of urea with no added Ca^{++}.

As in the case of the active Ca-trypsin complex, the active DBS-trypsin complex (17) may likewise be refractory to interaction with urea. It is pertinent to recall that several fatty acid anions have also been reported to exert a protective action on proteins against heat and urea denaturation (23).

SUMMARY

The loss in trypsin activity effected by 3.3 to 5.0 M urea at pH 7.6 is primarily the result of an enhanced rate of autolysis. Higher concentrations of urea, 6.7 and 8.3 M, produce an active, stable system. At pH 4, however, trypsin activity was unaffected by any level of urea ranging from 0 to 8.3 M. The presence of Ca^{++} raised the level of urea required for inactivation or stabilization. The presence of decylbenzene sulfonate or substrate likewise protected the enzyme from urea inactivation. Dilution of enzyme systems initially exposed to stabilizing levels of urea was followed by a rapid loss in activity. Possible explanations for these varied observations have been proposed.

BIBLIOGRAPHY

1. Haurowitz, F., Tunca, M., Schwerin, P., and Göksu, V., *J. Biol. Chem.*, **157**, 621 (1945).

2. Linderstrøm-Lang, K., Proteins and enzymes, Stanford (1952).
3. Lineweaver, H., and Hoover, S. R., *J. Biol. Chem.*, **137,** 325 (1941).
4. Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, **23,** 275, 289 (1940).
5. Okunuki, K., Hagihara, B., Matsubara, O., Nakayama, T., and Yanagida, M., *Symposia Enzyme Chem.*, Japan, **9,** 1 (1954).
6. Lineweaver, H., and Schwimmer, S., *Enzymologia*, **10,** 81 (1941–42).
7. Kunitz, M., *J. Gen. Physiol.*, **22,** 207 (1938).
8. Steinhardt, J., *J. Biol. Chem.*, **123,** 543 (1938).
9. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **17,** 151 (1933–34).
10. Schales, O., *Proc. Soc. Exp. Biol. and Med.*, **79,** 75 (1952).
11. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline enzymes, New York, 2nd edition (1948).
12. Kunitz, M., *J. Gen. Physiol.*, **30,** 291 (1947).
13. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **17,** 591 (1933–34).
14. Gorini, L., and Felix, F., *Biochim. et biophys. acta*, **11,** 535 (1953).
15. Bier, M., and Nord, F. F., *Arch. Biochem. and Biophys.*, **33,** 320 (1951).
16. Gorini, L., *Biochim. et biophys. acta*, **7,** 318 (1951).
17. Viswanatha, T., Pallansch, M. J., and Liener, I. E., *J. Biol. Chem.*, **212,** 301 (1955).
18. Cunningham, L. W., Jr., Tietze, F., Green, N. M., and Neurath, H., *Discussions Faraday Soc.*, **13,** 58 (1953).
19. Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O., *Chem. Rev.*, **34,** 157 (1944).
20. Duke, J. A., Bier, M., and Nord, F. F., *Arch. Biochem. and Biophys.*, **40,** 424 (1952).
21. Simpson, R. B., and Kauzmann, W., *J. Am. Chem. Soc.*, **75,** 5139 (1953).
22. Green, N. M., and Neurath, H., *J. Biol. Chem.*, **204,** 379 (1953).
23. Duggan, E. L., and Luck, J. M., *J. Biol. Chem.*, **172,** 205 (1948).

By CH

Evi
larly t
of dif
histor
demo
Eadie
Mirsk
and I
with
differ
Meth
tones
ion e
purifi
lysoz
mato
acid
were
could
achie
gradu
salt
histo
repor
the l

* A
Amer
[1] W
catin
of his
gate
this t

XUM

CHROMATOGRAPHIC FRACTIONATION OF CALF THYMUS HISTONE*

By CHARLES F. CRAMPTON, STANFORD MOORE, AND WILLIAM H. STEIN

(From the Laboratories of The Rockefeller Institute for Medical Research, New York 21, New York)

(Received for publication, December 23, 1954)

Evidence has recently accumulated that histone preparations, particularly those derived from the nuclei of calf thymus, may contain a mixture of different basic proteins. Variations in the amino acid composition of histone fractions extracted and precipitated by different means have been demonstrated by Stedman and Stedman (2), Daly, Mirsky, and Ris (3), Eadie and Leaf (4), Hamer (5), Davison and Butler (6), and Daly and Mirsky (7). Electrophoretic heterogeneity has been shown by Grégoire and Limozin (8) and Butler, Davison, James, and Shooter (9, 10). Studies with the ultracentrifuge have indicated that various fractions possess different sedimentation rates ((9, 10), Ahlström (11), Luck *et al.* (12)). Methods which permit the isolation of individual, well characterized histones from the mixture have not been available, however. The fact that ion exchange chromatography has been employed successfully for the purification of basic proteins such as cytochrome *c* (13), ribonuclease (14), lysozyme (15), and chymotrypsinogen (16) prompted an attempt to chromatograph histones in a similar manner. Initial trials with the carboxylic acid resin IRC-50 in the sodium form were not successful. The histones were bound much more strongly than the proteins previously studied and could not be eluted unless very alkaline eluents were used.[1] In order to achieve increased eluting power, calcium and barium acetate buffers of gradually increasing ionic strength were employed with the corresponding salt of the resin. Some, but not all, of the components of calf thymus histone could be chromatographed under these conditions. The present report describes the purification and properties of the proteins comprising the less basic half of crude calf thymus histone.

* A preliminary report of this work was presented before the 126th meeting of the American Chemical Society, New York, September, 1954 (1).

[1] We are indebted to Dr. M. M. Daly and Dr. A. E. Mirsky for information indicating that the published IRC-50 procedures were not applicable to the fractionation of histones. The negative results with the sodium salt of the resin led us to investigate modifications which might extend the scope of the method to basic proteins of this type.

XUM

EXPERIMENTAL

Calf Thymus Histones—Preparations of the total histone mixture used in the present study were obtained by a previously described modification (17) of earlier methods in which Hammarsten's procedure is employed for dissociating histone from nucleic acid with NaCl and ethanol. The protein fraction thus prepared contained 17.4 per cent N (Kjeldahl) and 1.93 per cent S (Parr bomb) on an ash- and moisture-free basis (ash, 1.28 per cent). A sample of calf thymus histone isolated by dissociating the proteins from nucleic acid by extraction with 0.2 N HCl by the method of Mirsky and Pollister (18) was also used in some studies.[2]

Preparation of Tissue Extracts—In order to minimize the possibility that the chromatographic results obtained with the isolated histones might arise from artifacts formed during their preparation, crude tissue extracts were also chromatographed. These were obtained in one step by homogenizing thymus tissue with barium acetate and adding ethanol in sufficient quantity to precipitate barium nucleates and most neutral proteins. Fresh tissue was brought from the slaughter-house in cracked ice and processing was begun within 90 minutes of the death of the animal. The extractions were carried out at 4°. The thymus was trimmed and 10 gm. portions were homogenized in a Waring blendor with 90 ml. of water for 60 seconds. Enough solid $Ba(OAc)_2$ (15.3 gm.) was added to make the homogenate 0.6 M in Ba.[3] The mixture was allowed to stand for 30 minutes, and 2 volumes of 95 per cent ethanol were added in two equal portions with vigorous shaking. After an additional 30 minutes, the resulting fibrous precipitate was lifted from the mixture and squeezed until compact with the aid of a porcelain spatula in order to express the occluded fluid. After filtration to remove a granular precipitate, the filtrate (260 ml.) containing the histones was taken to dryness on a rotary evaporator (19), the bath temperature of which was kept below 40°. The residue was dissolved by the addition of 520 ml. of water, and an aliquot of 6 ml. was removed at this point for chromatographic analysis. The sample was adjusted to pH 6.7 with 2 N HOAc and centrifuged before being chromatographed. The remaining extract was dialyzed (27/32 inch NoJax casing) against cold running tap water for 24 hours and against distilled water at 4° for 18 hours. The solution was centrifuged and lyophilized. The yield was 450 mg. of material per 10 gm. of thymus tissue (wet weight).

[2] Calf thymus histone, lot No. 532, from the Worthington Biochemical Corporation, Freehold, New Jersey.

[3] Separate experiments showed that 0.6 M $Ba(OAc)_2$-ethanol caused the dissociation of a maximal amount of ninhydrin-positive material from isolated nucleohistone, 85 per cent of the ninhydrin color of the nucleohistone being recovered in the ethanolic extract.

A number of dialyzed and lyophilized 2.6 M NaCl-ethanol extracts of calf thymus homogenates were prepared exactly as described above except that enough NaCl (15.2 gm.) to render the extract 2.6 M was added to the aqueous homogenate instead of the $Ba(OAc)_2$.

Chromatography—The sodium salt of IRC-50 (XE-64) was prepared in the manner already described (14) and screened through a 200 mesh sieve (16, 20). The acid form of the resin was washed on a Büchner funnel with 2 M $Ba(OAc)_2$ (or $Ca(OAc)_2$) until the effluent was about pH 6.5 (pH 6.0 when Ca was used). The bed was washed with water, followed by several bed volumes of 0.1 M $Ba(OAc)_2$ buffer of pH 6.7[4] or 0.13 M $Ca(OAc)_2$ buffer of pH 6.2. The resin was suspended in the 0.1 M buffer and transferred to a 4 × 30 cm. chromatograph tube. For convenience in the final washing about 6 liters of buffer per 4 × 15 cm. resin bed were passed through over a 24 to 48 hour period to bring the pH of the effluent to the same value as that of the influent. The final equilibrations and the chromatography were performed at about 25°. Before reuse, the resin was cycled through the acid and sodium forms.

The analytical chromatograms were performed with columns 0.9 cm. in diameter and of the desired height, prepared from suspensions of the resin in the given buffer by the procedures already described (14, 21). Chromatograph tubes with sealed in filter plates (20) were employed.[5] To promote regular flow of solvent, a roll of silver gauze was inserted into the delivery tip of the chromatograph tube (14).

The sample to be analyzed contained 5 to 10 mg. of histone in 1 to 6 ml. of the initial buffer. Before the sample solution was placed on the column, an aliquot was removed and analyzed by the ninhydrin method (19) to permit subsequent calculation of the per cent recovery of ninhydrin color in the chromatographic analysis. Chromatography was carried out with eluents of gradually increasing ionic strength through the use of a small magnetically stirred mixing device (*cf.* (20)). In most instances the mixing chamber containing the buffer of lower ionic strength was made from a 30 ml. flat bottomed flask attached to the upper joint of a wash bottle, the assembly having a total capacity of about 50 ml. Changes in the rate at which the ionic strength of the eluent was increased were effected by altering the strength of the solution in the separatory funnel

[4] The buffer was prepared by adding 0.67 ml. of glacial acetic acid to 10 liters of 0.1 M $Ba(OAc)_2$. The stronger buffers were prepared with similar proportions. All pH values were checked with the glass electrode. Since the chromatographic behavior of the histones proved to be much more dependent on the Ba^{++} concentration than on pH, the relatively low buffering capacity of the eluents has not caused the results to vary unduly.

[5] It has been noted that silicone grease and glass cloth both adsorb appreciable quantities of histone. Adsorption by the sintered disk seems not to occur, however.

feeding into the mixing chamber. In order to minimize upward flow of the less dense buffer, a capillary was inserted between the two chambers (20) and the stop-cock of the separatory funnel was only partly opened. The columns could be operated at flow rates of from 1.5 to 9 ml. per hour without affecting their performance. The effluent was usually collected in 1 ml. fractions which were analyzed by the photometric ninhydrin method with 1 ml. of the modified ninhydrin reagent (22). The blank readings gradually increase as the $Ba(OAc)_2$ concentration in the effluent rises. The base-line for each peak on the effluent curve is estimated from the readings immediately preceding or following the peak.

The preparative scale experiments were carried out with a load of about 400 mg. of protein on columns 4 cm. in diameter, a 1 liter mixing chamber being employed. Since the cross-sectional area of the column is about 20-fold that of the columns 0.9 cm. in diameter, the effluent volumes at which the changes in eluent were made (and at which the peaks emerged) in the analytical scale chromatograms were multiplied by 20. The effluent was collected in 15 ml. fractions at a rate of 75 ml. per hour, and 0.5 ml. aliquots were pipetted for ninhydrin analysis. Fractions corresponding to a given protein peak were pooled and transferred to Visking casings for dialysis against cold running tap water and distilled water at 4°. After dialysis, the histones were obtained by lyophilization.

Determination of Amino Acid Composition of Isolated Histones—Samples of the lyophilized preparations were simultaneously weighed for the determination of moisture (dried at 100° over P_2O_5 for 3 hours at 0.1 mm. pressure), ash (residue on ignition), nitrogen (Kjeldahl), and amino acid composition. For hydrolysis, the samples (about 2 mg.) were heated at 110° with 6 N HCl in evacuated, sealed tubes for 22 and 70 hours in the manner already described for the analysis of ribonuclease by Hirs *et al.* (23). The amino acid analyses were performed by the use of 150 cm. columns of Dowex 50-X4 (20). An approximate value for tryptophan was obtained, after alkaline hydrolysis, by chromatography on starch columns with 0.1 N HCl as eluent (*cf.* (23)).

Results

Chromatography of Preparations of Thymus Histone—Attempts were first made to chromatograph the histone fraction on columns of sodium IRC-50. As eluents, sodium acetate buffers (pH 6.4) varying in ionic strength from 0.2 to 4 M were tried. The results indicated that more than 90 per cent of the histone preparation was being held tenaciously by the resin. When greater eluting power was achieved by the use of calcium ions, a series of peaks was obtained as illustrated by the effluent curve shown in Fig. 1. The resin was equilibrated initially with 0.13 M calcium

acetate at pH 6.2. Previous test-tube experiments (for the procedure see Hirs *et al.* (14)) had shown that at this calcium concentration the resin binds nearly all of the histone in the dialyzed preparations from calf thymus. In contrast to the results obtained with sodium, however, higher concentrations of calcium proved able to elute portions of the bound histone. Thus, as the concentration of calcium acetate in the influent buffer was gradually increased from 0.13 toward 2 M, three principal peaks were eluted, accounting for 4, 24, and 20 per cent of the total ninhydrin color applied to the column. Even if the experiment was continued to 150 effluent ml., or if the concentration of the influent buffer was changed

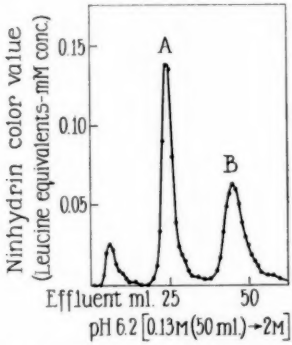


FIG. 1. Chromatography of calf thymus histones (5 mg.) on Ca IRC-50 (column 0.9 × 15 cm.). The ionic strength of the influent was gradually increased by allowing 2 M buffer at pH 6.2 to flow into a 50 ml. mixing chamber containing initially a 0.13 M buffer at the same pH. Histone Fractions A and B accounted for 24 and 20 per cent, respectively, of the total ninhydrin color added to the column.

directly to 2 M, no further peaks appeared.[6] No more than about 50 per cent of the starting material (on a ninhydrin basis) could be recovered from the chromatogram. Similar results were obtained with a commercial sample[2] of histone chloride.

The low yield does not result from the irreversible binding by the resin of major quantities of Fraction A or B, because, when a portion of the material isolated from Peak A was rechromatographed under the same conditions, it gave a single peak in the original position in 88 per cent yield. Similarly, material from Peak B, upon rechromatography, gave at

[6] If the Ca concentration of the initial buffer is above 1 M, a much greater percentage of the ninhydrin color is recovered in a single unretarded peak at the column volume. Thus, there is a part of the histone mixture which, once it has been bound by the resin, can no longer be eluted by a buffer possessing an ionic strength sufficiently high to have prevented binding of this histone fraction initially.

its original position a single peak in a yield of 83 per cent. Apparently the low yield results from the presence in the histone preparation of additional components that are not eluted from the resin under these conditions. It is probable that the fraction which is retained is composed of proteins more basic than Fraction B, which, in turn, is more basic than Fraction A. This supposition is in accord with the results of the amino acid analyses subsequently obtained in the present study. The fact that the positions of the peaks did not change upon rechromatography supported the conclusion that the substances obtained were not artifacts induced by the column and that competition and displacement effects were not playing a rôle in the fractionation process. Thus, for the proteins comprising the least basic half of the histone mixture, the IRC-50 columns appeared to provide a suitable means of fractionation.

When further study was made of the variables which determine the resolving power of the system, it was found that the results were not dependent upon the flow rate (up to 15 ml. per hour per sq. cm. of cross-sectional area of the column), upon the temperature in the range from 4–25°, or upon the pH in the range from pH 6.0 to 7.5. The chromatographic behavior of histones on calcium IRC-50 is thus markedly less sensitive to changes in the pH of the eluent than was found to be the case for the basic proteins previously studied with the sodium form of the resin (14–16). If the height of the column was doubled, but the rate of change of calcium concentration in the influent kept the same, the separation of Fractions A and B was improved. A less rapid rate of change in the composition of the influent buffer also yielded better separation, but was accompanied by a broadening of the peaks which approached impractical limits (zones 50 to 100 ml. wide) when the rate of change was reduced to one-fifth of that used for the experiment shown in Fig. 1.

Barium acetate, with the barium form of the resin, was found to possess stronger eluting power than the corresponding calcium buffer and was, therefore, adopted for the subsequent chromatograms.

The data obtained thus far do not provide decisive information as to the mechanisms operating to produce the separations. When ribonuclease is chromatographed on sodium IRC-50, the pH and ionic strength are maintained constant, and there can be no doubt that a true reversible distribution of the protein between the buffer and the resin phase occurs (14, 24). To chromatograph histones, however, a changing concentration of Ca or Ba in the influent buffer is necessary. The effluent does not reflect exactly the change in the influent because the IRC-50 column is not quite saturated and continues to take up small amounts of Ca^{++} or Ba^{++} from the buffer as the cation concentration is raised. Therefore, in order to determine whether the protein has a finite distribution coefficient between

buffer and Ca or Ba IRC-50, it will be necessary to perform single stage, test-tube experiments at constant pH and ionic strength with purified histone fractions (*cf.* (14)).

Chromatography of Extracts of Tissues and Isolated Nuclei—In Fig. 2, the chromatographic behavior (with the barium system) of a total histone preparation isolated by the modified Hammarsten procedure (17) is compared with that of a tissue extract prepared by the simple one-step $Ba(OAc)_2$-ethanol precipitation described in the experimental section.

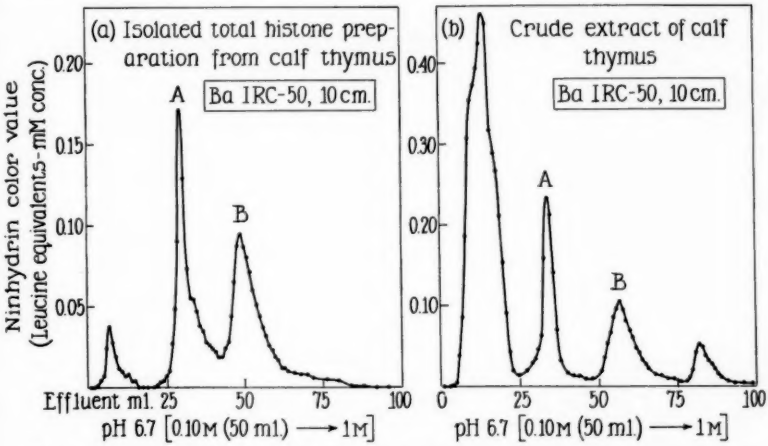


FIG. 2. Comparison of the chromatographic behavior of a total histone preparation (*a*) with that of a crude extract of the whole tissue prepared by a single step barium acetate-ethanol precipitation (*b*). Dialysis of the extract used in (*b*) removed the constituents responsible for the large peak before histone Fraction A and for the smaller peak after histone Fraction B.

Two peaks, A and B, appear at nearly the same positions on each chromatogram. The insensitivity of the IRC-50 columns to salts and other constituents in tissue extracts (14) makes feasible the chromatographic analysis of the crude histone solution. The peaks preceding and following Peaks A and B in Fig. 2, *b* largely disappear if the crude tissue extract is dialyzed prior to chromatography.[7]

Chromatography of a number of dialyzed and lyophilized 2.6 M NaCl-ethanol extracts consistently showed the presence of major components, the behavior of which was similar to that of Fractions A and B. If the

[7] Under the conditions of the chromatograms, all of the amino acids present in a mixture simulating the composition of a hydrolysate of bovine serum albumin have emerged by 25 effluent ml.

XUM

aqueous homogenate was allowed to autolyze for 4 hours at 29° prior to extraction with NaCl-ethanol, the chromatographic picture given by the final preparation was qualitatively the same as that shown in Fig. 2, *b*, except that the amounts of Fractions A and B were less. Autolysis of the extract did not lead to increased heterogeneity.

Calf thymus nuclei, which had been isolated by a modified Behren's technique (Allfrey, Stern, Mirsky, and Saetren (25)[8]), were extracted with 0.6 M $Ba(OAc)_2$-ethanol and the extract was chromatographed under the same conditions that were used for the experiment illustrated in Fig. 3.

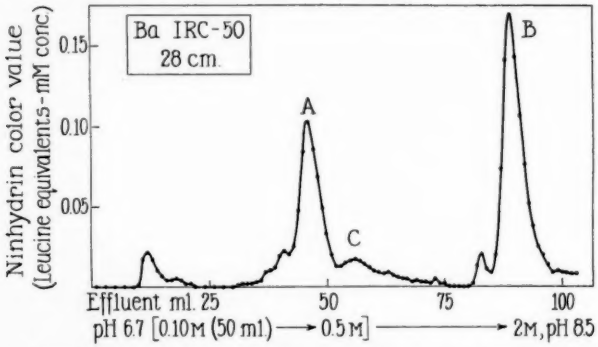


FIG. 3. Chromatography of histones (5 mg.) from calf thymus on Ba IRC-50 (column 0.9 × 28 cm.). The resolving power is improved over that shown in Fig. 2 by using a longer column and by making the initial increase in the strength of the buffer more gradual. A 0.5 M barium acetate buffer was allowed to flow into the 50 ml. mixing chamber until 54 effluent ml. were collected, and a 2 M buffer (pH 8.5) was then substituted in order to increase the rate of elution of histone Fraction B. The effluent did not become more alkaline than pH 7.4 because of the very low buffering capacity of the $Ba(OAc)_2$ buffer at pH 8.5.

Components emerged which corresponded both in position and relative amounts to histone Fractions A, B, and C present in total histone preparations.

The results obtained indicate that the histone Fractions A, B, and C probably preexist in the nuclei of the gland and are not formed as a result of enzymatic action or other changes occurring during the preparation of the histones.

Isolation of Histone Fractions A, B, and C—In the preparative scale experiments and in the analytical scale chromatograms that served as models, a longer column (28 cm.) was employed and the change in the cat-

[8] We are indebted to Dr. A. E. Mirsky and Dr. V. Allfrey, who, through a generous gift of isolated nuclei, prompted us to perform this experiment.

ion concentration in the influent was made more gradually. The results of such an analysis are shown in Fig. 3. The increased resolution observed in this experiment was fully reproducible when the same histone preparation was analyzed and was also obtained with numerous total histone preparations isolated in a similar manner. In addition to the major Peaks A and B, the two smaller peaks before and after Peak A were found repeatedly. The least basic half of crude calf thymus histone contains, therefore, five or more chromatographically differentiable proteins.

In an experiment paralleling the one shown in Fig. 3, the proteins responsible for three of the peaks (A, B, and C) were isolated from the effluent after chromatography of 400 mg. of total histone on a column 4 cm. in diameter. The pooled fractions corresponding to only the central part of Peak A were taken, representing 13 per cent of the total ninhydrin color added to the column. The yield of dialyzed, lyophilized protein was 21 mg. In order to obtain information as to the homogeneity of Peak B, independent amino acid analyses were performed on samples taken from the left-hand half and the right-hand half of the peak. The second portion taken corresponded to 11 per cent of the total ninhydrin color and yielded 39 mg. of Fraction B. A sample of Fraction C corresponding to 4 per cent of the ninhydrin color gave 8 mg. of histone. During the removal of the $Ba(OAc)_2$ by dialysis there were losses of 6, 19, and 1 per cent for histone Fractions A, B, and C, based on the recovery of ninhydrin color. From the weight of the histone obtained, and the per cent of the total ninhydrin color in each of the peaks on the curve, it is calculated that, on a weight basis, the proteins in Fractions A, B, and C represent a minimum of approximately 10, 22, and 3 per cent of the histone preparation. The sizes of the peaks on the effluent curve in Fig. 3 convey the relative amounts of proteins on a ninhydrin color basis but not on a weight basis, since the individual proteins have different ninhydrin color values per mg., as a result of differences in amino acid composition.

Fraction A contained 17.95 per cent N (Kjeldahl) corrected for moisture and for 2.08 per cent ash. Fraction B similarly contained 16.78 per cent N and 7.86 per cent ash on ignition. The ash content probably could have been reduced by redialysis. Fraction C, which was not isolated in sufficient quantity for moisture and ash determinations, gave 13.99 per cent N on the lyophilized but not dried preparation.

Amino Acid Composition of Isolated Histones—In order to characterize further the proteins responsible for Peaks A, B, and C in Fig. 3, amino acid analyses of the isolated fractions were performed. It was also hoped to obtain in this manner additional evidence as to the homogeneity of the fractions.

The analytical data obtained by chromatographing 22 and 70 hour hy-

TABLE I

Amino Acid Composition of Hydrolysates of Calf Thymus Histone and Derived Fractions

The compositions were determined by chromatography of the 6 N HCl hydrolysates on columns of Dowex 50-X4 (17, 20). Analysis of an alkaline hydrolysate showed that the total histone mixture contained less than 0.08 per cent of tryptophan.

Amino acid	N as per cent of total N							
	Total histone mixture	Histone Fraction A		Histone Fraction B			Histone Fraction C	
				Sample I*	Sample II*			
	Hydrolysis for							
	22 hrs.	22 hrs.	70 hrs.	22 hrs.	22 hrs.	70 hrs.	22 hrs.	70 hrs.
Aspartic acid	3.10	1.52	1.52	3.60	3.52	3.53	2.30	2.15
Glutamic "	5.31	2.65	2.62	5.49	5.35	5.62	3.73	3.86
Glycine	5.44	4.99	5.19	5.37	5.24	5.49	5.17	4.71
Alanine	8.75	17.80	17.66	7.49	7.46	7.67	13.32	12.55
Valine	3.98	3.77	3.81	4.16	4.30	4.33	3.31	2.97
Leucine	5.02	3.41	3.31	5.49	(5.05)†	5.42	4.02	4.06
Isoleucine	2.82	0.77	0.77	2.83	2.95	2.95	1.63	1.52
Serine	3.13	4.25	2.94	4.04	3.83	2.49	3.64	2.34
Threonine	3.41	3.81	3.33	3.20	3.00	2.44	3.49	2.84
Cystine	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Methionine‡	0.63	<0.06	<0.06	0.51	0.58	0.62	0.29	0.29
Proline	3.23	6.79	6.67	2.39	2.89	2.64	4.70	4.20
Phenylalanine	1.36	0.42	0.47	0.98	0.86	0.91	1.05	0.77
Tyrosine	1.53	0.45	0.38	1.77	1.92	1.69	1.10	0.73
Histidine	3.87	<0.49	<0.15	5.03	4.80	4.90	(0.91)§	2.07
Lysine	19.11	39.27	36.09	15.81	(15.85)†	16.97	24.92	24.68
Arginine	21.47	‖	6.74	18.60	19.15	19.76	11.97	12.65
Ammonia	4.99	2.74	4.51	6.56	5.51	8.39	3.54	5.82

* The analyses were performed on samples pooled from the left side (Sample I) and right side (Sample II) of the histone B peak on the effluent curve. The agreement among the analytical values is supporting evidence that histone Fraction B is chromatographically homogeneous. The values in Table II are based on the analyses of Sample II.

† The values for leucine and lysine in Table II are based on the results obtained for the 70 hour hydrolysate of histone Fraction B. These results were confirmed by a duplicate analysis of a 70 hour hydrolysate, which yielded values of 5.64 for leucine and 16.77 for lysine.

‡ The methionine figures include the 10 per cent correction for losses during chromatography (20).

§ The 70 hour histidine value was used for the calculations in Table II. Further analyses would be required to verify whether the rate of liberation of histidine is actually slow in this case.

‖ The analysis was lost.

drolysates of Fractions A, B, and C on columns of Dowex 50 (20, 23) are given in Table I, together with an analysis of the initial total histone preparation. Hydrolysis for two different lengths of time has been shown by several earlier investigations (23, 26–28) to permit more accurate estimates both of those amino acids that decompose detectably on acid hydrolysis and of those that may not have been fully liberated after the shorter time. The results in Table I are expressed in terms of amino acid nitrogen as per cent of total nitrogen and are thus independent of the nature and the quantity of any anion that might be present in the various preparations. If, as seems likely, these very basic proteins do bind anions, such as acetate, the presence of 1 acetate per ϵ-amino group of lysine and per guanido group of arginine would contribute as much as 10 to 15 per cent to the weight of the preparation. Results expressed in terms of amino acid nitrogen as per cent of total nitrogen are also independent of the ash content of the various preparations. The ash content of Fraction B was 7.86 per cent after ignition (presumably to BaO), but this figure does not take into account the mode of combination of the metal ion. The contribution of the ash to the weight of the original preparation would be different, depending upon whether the metallic cation existed as the salt of the free carboxyl groups of aspartic and glutamic acids or as barium acetate.

In Table II are given the amino acid compositions of Fractions A, B, and C (in terms of amino acid N as per cent of total N) derived from the analytical data in Table I. The values for serine, threonine, and tyrosine, and for proline in Fractions B and C have been calculated by extrapolating the results for the 22 hour and 70 hour hydrolysates to zero time, on the assumption that the decomposition follows first order kinetics in each case (*cf.* (23)). Leucine and lysine in Fraction B appear to be the only amino acids incompletely liberated after 22 hours of hydrolysis. The values for 70 hours have, therefore, been used in Table II. The remaining figures in Table II are averages of the values obtained for the 22 and 70 hour hydrolysates. Tryptophan has been assumed to be absent from Fractions A, B, and C, inasmuch as a negligible amount (less than 0.08 per cent) of this amino acid was found in the total histone preparation by chromatography of an alkaline hydrolysate on starch (23). The amide-NH_3 values have been estimated from the NH_3 content of the 22 hour hydrolysate corrected for the NH_3 calculated to have arisen by the decomposition of serine and threonine in this period (23). As can be seen from the bottom of Table II, the amino acid analyses account for more than 90 per cent of the nitrogen of the preparations in each case. More accurate results and more complete recoveries might be possible if a more extensive series of analyses was performed.

XUM

The differences in the amino acid compositions of the three isolated fractions are readily apparent from the data in Table II. In Fraction A, lysine N represents 37.7 per cent of the total N of the protein, corresponding to over one-fourth of all of the amino acid residues. Alanine and

TABLE II

Amino Acid Composition of Calf Thymus Histone and Derived Fractions

Based on the analytical data in Table I.

Amino acid	N as per cent of total N			
	Total histone mixture	Histone Fraction A	Histone Fraction B	Histone Fraction C
Aspartic acid	3.10	1.52	3.53	2.23
Glutamic "	5.31	2.64	5.49	3.80
Glycine	5.44	5.09	5.37	4.94
Alanine	8.75	17.73	7.57	12.94
Valine	3.98	3.79	4.32	3.14
Leucine	5.02	3.36	5.42	4.04
Isoleucine	2.82	0.77	2.95	1.58
Serine	3.77*	5.05†	4.65†	4.47†
Threonine	3.70*	4.07†	3.29†	3.84†
Cystine	0	0	0	0
Methionine	0.63	0	0.60	0.29
Proline	3.23	6.73	3.02†	4.96†
Phenylalanine	1.36	0.45	0.89	0.91
Tyrosine	1.64*	0.48†	2.04†	1.33†
Histidine	3.87	0	4.85	2.07
Lysine	19.11	37.68	16.97	24.80
Arginine	21.47	6.74	19.46	12.31
Amide-NH_3	4.06	1.68	4.40	2.36
Total	97.26	97.78	94.82	90.01

* The values for serine, threonine, and tyrosine in the 22 hour hydrolysate of the total histone mixture were corrected for the average percentage losses of these amino acids found for histone Fractions A and B.

† Values obtained from the data in Table I by extrapolation, based on the assumption that the decomposition of the amino acids follows first order kinetics (*cf.* (23)).

proline (accounting for 17.7 and 6.7 per cent of the total N, respectively) are unusually abundant, and four amino acids are absent, namely cystine, methionine, histidine, and tryptophan. The absence of these amino acids indicates that Fraction A has been separated from other histones that contain methionine and histidine.

The amino acid composition of Fraction B is much closer to that of the total histone than is that of Fraction A. With the exception of glycine,

phenylalanine, arginine, and probably serine and methionine, the values for the total material are intermediate between those for Fraction A and those for Fraction B. Evidence favoring the homogeneity of Fraction B is provided by analyses of a sample from the front half (Sample I, Table I) and the back half (Sample II, Table I) of the peak obtained from the IRC-50 column. The analyses of the 22 hour hydrolysates agreed well, except for serine and threonine, which are subject to varying degrees of decomposition. If a mixture of proteins were emerging from the column, some difference should be detected in the amino acid composition of fractions collected from different portions of the peak. There is, for example, a great difference in the amino acid compositions of the two neighboring Fractions A and C (Table II). Fraction C contains a significant quantity of histidine, and only a little more than half the lysine of Fraction A, but twice the phenylalanine and tyrosine.

DISCUSSION

The data presented in this communication emphasize that major structural differences may exist among the proteins conventionally classified as histones. The amino acid analyses of Fractions A and B isolated from a thymus histone mixture by chromatography on IRC-50 indicate that two of these proteins may have been obtained in homogeneous form. Comparison of the arginine content of Fractions A, B, and C with that of the total histone mixture shows that the proteins which it has not so far been possible to chromatograph on IRC-50, and which are retained by the resin, must be higher in arginine content than the isolated fractions.[9]

Proteins or polypeptides rich in lysine have been isolated previously from calf thymus. Bloom, Watson, Cromartie, and Freed (29) obtained a "polylysine" fraction which inhibited the growth of the anthrax bacillus. Of the nitrogen of this preparation, 36.7 per cent was lysine N, 7.36 per cent was arginine N, and 1.84 per cent was histidine N. The complete amino acid composition was not determined. The lysine and arginine values are very similar to those of Fraction A. Because it contains no histidine, Fraction A clearly differs from the "polylysine" fraction of Bloom *et al.* Davison and Butler (6) have published preliminary amino acid analyses of a histone fraction of high lysine content prepared by fractionation of crude histone with ammonia and acetone (9) or by extraction of nucleoprotein with dilute citric acid (10). Daly and Mirsky (7) have described the isolation of lysine-rich histone fractions prepared by partial extraction of calf thymus nuclei with NaCl and dilute citric acid.

Hirsch and Dubos (30) have recently reported the isolation from calf

[9] It has not been possible thus far to chromatograph substances known to have a high arginine content, such as salmine, on columns of Ba IRC-50.

thymus of a basic peptide fraction with antimycobacterial activity. On analysis 23.8 per cent of the N of the preparation was lysine N and 30.3 per cent was arginine N. The components of this mixture were shown to be dialyzable, however, and hence would not have been included in the total histone preparations utilized in the present study.

The authors are greatly indebted to Miss Joyce F. Scheer for technical assistance and to Mr. S. Theodore Bella who performed the microanalyses reported in this paper.

SUMMARY

A total histone preparation from calf thymus has been fractionated by chromatography on columns of the carboxylic acid resin IRC-50. The material was bound firmly by the sodium form of the resin, but chromatography could be achieved when calcium or barium acetate buffers (pH 6.2 or 6.7) of continuously increasing ionic strength (0.1 to 2.0 M) were employed with the Ca or Ba form of the resin. Under these conditions, only the less basic half of the total histone mixture could be eluted. Even the increased eluting power of the Ca or the Ba system did not serve to elute the most basic components, which were still retained by the resin. The chromatograms showed that the portion of the total histone eluted was composed of five or more proteins. Three of these components have been separated chromatographically and isolated by dialysis and lyophilization. The three protein fractions, A, B, and C, represent a minimum of about 10, 22, and 3 per cent of the total mixture. Each preparation has been characterized by complete amino acid analyses by chromatography on columns of Dowex 50-X4. Component A is unusually rich in lysine and alanine (37.7 and 17.7 N as per cent of total N, respectively) and contains no cystine, methionine, histidine, or tryptophan. The results of the amino acid analyses, taken in conjunction with the behavior of the proteins on the columns of IRC-50, indicate that histone Fractions A and B may have been isolated in homogeneous form. The chromatographic procedure is applicable to crude extracts of calf thymus prepared by treatment of an aqueous homogenate with $Ba(OAc)_2$ and ethanol. In this manner evidence has been obtained that histones A and B are present in tissue extracts freshly prepared by a one-step fractionation procedure. The chromatographic technique may also provide a means for comparing the less basic histones obtained from various tissues and from different species.

BIBLIOGRAPHY

1. Crampton, C. F., Moore, S., and Stein, W. H., Abstracts, American Chemical Society, 126th meeting, New York, 90C, Sept. (1954).

2. Stedman, E., and Stedman, E., *Nature*, **166,** 780 (1950); *Phil. Tr. Roy. Soc. London, Series B*, **235,** 565 (1951).
3. Daly, M. M., Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **34,** 439 (1951–52).
4. Eadie, E. J., and Leaf, G., *Biochem. J.*, **50,** p. xxxiv (1952).
5. Hamer, D., *Brit. J. Cancer*, **7,** 151 (1953).
6. Davison, P. F., and Butler, J. A. V., *Biochim. et biophys. acta*, **15,** 439 (1954).
7. Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.*, **38,** 405 (1955).
8. Grégoire, J., and Limozin, M., *Bull. Soc. chim. biol.*, **36,** 15 (1954).
9. Butler, J. A. V., Davison, P. F., James, D. W. F., and Shooter, K. V., *Biochim. et biophys. acta*, **13,** 224 (1954); *Biochem. J.*, **57,** p. xxiv (1954).
10. Davison, P. F., James, D. W. F., Shooter, K. V., and Butler, J. A. V., *Biochim. et biophys. acta*, **15,** 415 (1954).
11. Ahlström, L., *Ark. Kemi, Mineral. o. Geol.*, **24 A,** No. 31 (1947).
12. Luck, J. M., Cook, H. A., and Eldredge, N., Abstracts, XIII International Congress of Pure and Applied Chemistry, Stockholm, 87 (1953).
13. Paléus, S., and Neilands, J. B., *Acta chem. Scand.*, **4,** 1024 (1950).
14. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **200,** 493 (1953).
15. Tallan, H. H., and Stein, W. H., *J. Biol. Chem.*, **200,** 507 (1953).
16. Hirs, C. H. W., *J. Biol. Chem.*, **205,** 93 (1953).
17. Crampton, C. F., Lipshitz, R., and Chargaff, E., *J. Biol. Chem.*, **206,** 499 (1954).
18. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30,** 117 (1946).
19. Craig, L. C., Gregory, J. D., and Hausmann, W., *Anal. Chem.*, **22,** 1462 (1950).
20. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211,** 893 (1954).
21. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192,** 663 (1951).
22. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211,** 907 (1954).
23. Hirs, C. H. W., Stein, W. H., and Moore, S., *J. Biol. Chem.*, **211,** 941 (1954).
24. Moore, S., and Stein, W. H., *Ann. Rev. Biochem.*, **21,** 521 (1952).
25. Allfrey, V., Stern, H., Mirsky, A. E., and Saetren, H., *J. Gen. Physiol.*, **35,** 529 (1952).
26. Harfenist, E., *J. Am. Chem. Soc.*, **75,** 5528 (1953).
27. Smith, E. L., and Stockell, A., *J. Biol. Chem.*, **207,** 501 (1954).
28. Smith, E. L., Stockell, A., and Kimmel, J. R., *J. Biol. Chem.*, **207,** 551 (1954).
29. Bloom, W. L., Watson, D. W., Cromartie, W. J., and Freed, M., *J. Infect. Dis.*, **80,** 41 (1947).
30. Hirsch, J. G., and Dubos, R. J., *J. Exp. Med.*, **99,** 65 (1954).

XUM

STUDIES ON THE RELATIONSHIPS BETWEEN INSULIN, GLUCOSAMINE, AND GLUCOSE IN RAT DIAPHRAGMS*

By HENRY I. NAKADA, TOSHIKO N. MORITA, AND ARNE N. WICK

(From the Scripps Metabolic Clinic, La Jolla, California)

(Received for publication, January 31, 1955)

One approach toward the elucidation of the mechanism of insulin action is to study the effect of insulin on glucose analogues. Studies of this type have been carried out in this laboratory by use of the eviscerated and nephrectomized rabbit. It has been shown (1), with the above test preparation, that insulin accelerates the transfer of D-glucosamine from the extracellular fluid into the cells. Concurrently, the demand for glucose by the maximally insulin-treated animal was lowered considerably after a single injection of D-glucosamine. Since the decrease in glucose requirements of these animals could not be explained, even assuming complete conversion of D-glucosamine to glucose, it was suggested that D-glucosamine acts in some manner as a metabolic block.

While the metabolic changes observed in the eviscerated animal are usually attributed to the muscle cells, it is desirable to test this directly. Consequently, we have examined the effect of insulin action on glucosamine, using the isolated rat diaphragm. Studies of this type can be carried out in the absence of glucose, in contrast to the intact animal, in which glucose is always available to the muscle tissues.

Methods

Tissue Preparation—Young fed rats (Slonaker strain) were decapitated, and the diaphragms were rapidly removed and chilled in cold Krebs-Ringer-phosphate buffer solution (pH 7.4). This medium was used in all diaphragm experiments. The tissues were trimmed, divided, blotted, and weighed. For studies on the effect of insulin[1] on sugar uptake, the hemidiaphragms were divided between the control and insulin-containing flasks. When the effect of glucose competitors was studied, each of about six diaphragms was divided nearly equally among the six vessels in each series. The diaphragms were added to chilled Warburg vessels previously prepared

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

[1] The crystalline zinc insulin used in this work was contributed by the Lilly Research Laboratories.

XUM

with the necessary substrates and medium. The final volume in all experiments was 3 ml., and 0.2 ml. of KOH (20 per cent) was added to the center wells to absorb CO_2. The flasks were gassed for 5 minutes with oxygen, then incubated for 2 hours at 37°. When radioactive substrates were used, 0.2 ml. of 50 per cent H_2SO_4 was tipped in from the side arm to stop the reaction and to liberate CO_2. At least 10 minutes were allowed for absorption of the CO_2. The contents and rinses from the center well were transferred to small Erlenmeyer flasks, 0.4 mmole of carrier Na_2CO_3 added, and the carbonates precipitated as $BaCO_3$. The $BaCO_3$ was collected by filtration, dried, and assayed for radioactivity.

Determination of Glucose and Glucosamine—After incubation the flasks were removed, and the diaphragms were quickly separated from the medium and rinsed three times. The combined medium and rinses were made up to 20 ml., and aliquots were immediately pipetted into test-tubes containing the reagents for reducing sugar or glucosamine determinations. The controls with tissue incubated without substrate gave negligible values for reducing sugar or glucosamine. Reducing sugars were determined by the method of Miller and Van Slyke (2) and glucosamine by a modification of the Elson and Morgan colorimetric method (1).

When both glucose and glucosamine were present in the flasks, the glucose was determined after separation on a cation exchange column. In these experiments, 1 ml. of the medium was transferred immediately after incubation to Duolite C-3 columns (8 × 70 mm.) regenerated to the acid form with HCl. Glucose was washed out with water at a rate of approximately 2 ml. per minute until 25 ml. were collected. Glucose recoveries by this method were between 96 and 100 per cent. Control glucose determinations were run for added glucose and column glucose recovery with each experiment.

Results

As shown in Table I, D-glucosamine disappears from the medium when incubated with rat diaphragms, and this disappearance is accelerated on addition of insulin. The amino sugar uptake by the diaphragms was measured by difference in the quantity of sugar present at zero time and at the end of 2 hours of incubation. Determinations made by both a reducing method and the glucosamine color reaction of Elson and Morgan showed a close agreement between the two methods.

It was found that in the eviscerated rabbit D-glucosamine retarded the entry of glucose into the cell. This blocking action of glucose by glucosamine has also been demonstrated in the isolated diaphragm (Table II). At a glucose-glucosamine concentration of 0.01 to 0.005 M, respectively, glucose uptake was reduced to about one-half the value obtained in the absence of glucosamine.

TABLE I

Effect of Insulin on Glucosamine and Glucose Disappearance by Rat Diaphragm

Experiment No.	Substrate	Sugar disappearance, mg. per gm. wet weight			
		As reducing sugar		As glucosamine by colorimetric determination	
		Without insulin	With insulin	Without insulin	With insulin
1	D-Glucosamine	0.801	3.41	0.55	3.65
2	"	1.54	4.06	1.52	4.03
3	"	1.97	3.43	2.13	3.36
4	"	0.963	3.14	1.13	3.42
5	"	1.30	2.99	1.59	2.91
6	"	2.09	3.34		
7	"	1.67	3.06		
8	"	2.10	2.84		
9	D-Glucose	4.42	5.63		
10	"	3.42	5.12		
11	"	4.47	5.67		

Two flasks were used in each experiment, containing paired hemidiaphragms from two rats (trimmed to approximately 350 mg. of wet weight). The final sugar concentration was 0.01 M, and insulin, when used, was 0.7 unit per flask. The flasks were incubated at 37° for 2 hours with O_2 in the gas phase.

TABLE II

Effect of Glucosamine on Glucose Uptake by Rat Diaphragm

Experiment No.	Glucose disappearance, mg. per gm. wet tissue	
	No glucosamine	Glucosamine added
1	4.30	2.16
2	5.03	2.66
3	5.10	3.59
4	4.19	2.55

The two flasks in each experiment contained paired hemidiaphragms from two rats (trimmed to approximately 350 mg. of wet weight) suspended in 3 ml. of medium consisting of Krebs-Ringer-phosphate buffer, pH 7.4, and the substrates which were at a final concentration of 0.01 M for glucose, 0.005 M for glucosamine, and 0.7 unit of crystalline zinc insulin per flask. The flasks were incubated at 37° for 2 hours with O_2 in the gas phase. Glucose was determined after removal of glucosamine by passage through cation exchange columns.

This reduction of glucose uptake in the presence of glucosamine is reflected in the amount of glucose converted to CO_2. Using a constant concentration of C^{14} uniformly labeled glucose in the presence of increasing concentrations of D-glucosamine, we found that glucose oxidation by rat

diaphragms was decreased significantly (Fig. 1). This apparent inhibition of glucose oxidation is not due to ammonia formation or the increased chloride ion concentration, for ammonium chloride had relatively little effect on glucose oxidation by this system. Fructose was also tested as an inhibitor for glucose oxidation, but its effect is not as great, especially at lower concentrations, as that of glucosamine. It is noteworthy that, at a

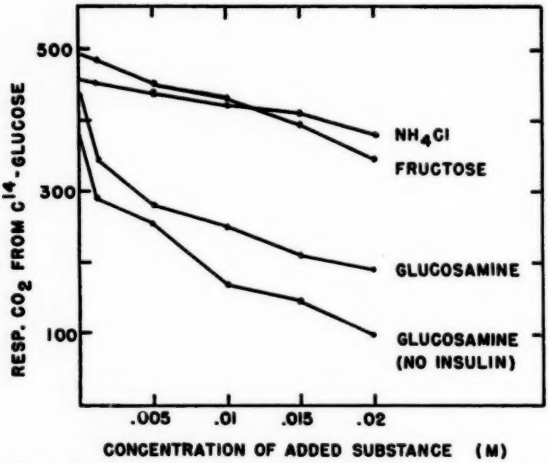


FIG. 1. Effect of added substances on glucose oxidation by rat diaphragm. For each experiment, the diaphragms from six rats were divided nearly equally among the six flasks. The weights of diaphragms in each vessel were adjusted to 350 ± 10 mg. Each flask contained radioactive uniformly labeled glucose (0.01 M) (specific activity 18,767 c.p.m. at infinite thickness as $BaCO_3$), 0.7 unit of insulin (except where noted), as well as the designated amounts of D-glucosamine hydrochloride, NH_4Cl, or D-fructose. Incubations were carried out at 37° for 2 hours with oxygen in the gas phase. Activity was calculated as (counts per minute of $BaCO_3$ at infinite thickness × micromoles of carbon)/(gm. of wet tissue).

glucose to glucosamine ratio of 2:1, the extent of inhibition is about the same for glucose uptake (Table II) and glucose oxidation (Fig. 1). The action of glucosamine in reducing the conversion of glucose to CO_2 in the absence of insulin is identical with its action in the presence of insulin, except for the correspondingly lower rates of glucose oxidation. The effect of preincubating glucosamine with the diaphragms for 10 minutes, then adding the radiocarbon glucose, resulted in respiratory CO_2 values that were indistinguishable from those in experiments in which both substrates were present from the start.

DISCUSSION

In these studies with the isolated rat diaphragm, glucosamine has been shown to behave in a manner similar to that reported for eviscerated rabbits (1). Glucosamine disappears from the incubation media, and this disappearance is definitely enhanced in the presence of insulin. The uptake of glucosamine was determined both by sugar reduction and by the glucosamine colorimetric method. The close agreement between the two methods indicates the simultaneous disappearance of the amino group and the aldehyde group of glucosamine and not merely the masking of the amino group or its removal by deamination.

Mackler and Guest (3) have reported evidence for the utilization of fructose by the rat diaphragm that is independent of the insulin-controlled glucose-utilizing system. The straight line oxidation curve obtained (Fig. 1) when fructose at various concentrations was superimposed on radioactive glucose shows that fructose was competing with glucose oxidation only to a small degree.

On the other hand, glucosamine was found to compete vigorously with glucose, especially at low concentrations of glucosamine. The site of this competition with glucose can occur at any of the glycolytic enzymes that act on both of these sugars. Harpur and Quastel (4) as well as other workers (5–7) have demonstrated the phosphorylation of glucosamine by hexokinase and adenosine triphosphate. Hoare and Kerly (6) have reported that retinal extracts could not metabolize glucosamine beyond the glucosamine-6-phosphate stage, while glucose or fructose could be metabolized further, and Brown (8) has shown that the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate is catalyzed by purified rabbit muscle phosphoglucomutase.

The fact that glucosamine not only depresses glucose oxidation, but also lowers glucose uptake and can respond to insulin, can be interpreted as a competitive action between glucosamine and glucose for entry into the cell, the mechanism suggested (9, 10) as the site of insulin action.

SUMMARY

1. Insulin increases the disappearance of glucosamine when incubated with rat diaphragm.

2. Glucosamine inhibits the uptake of glucose by the rat diaphragm.

3. Glucose oxidation is decreased more by glucosamine than by fructose or ammonium chloride when these are incubated with rat diaphragm.

These results suggest that glucosamine competes with glucose at the site of insulin action.

BIBLIOGRAPHY

1. Wick, A. N., Drury, D. R., Nakada, H. I., Barnet, H. N., and Morita, T. N., *J. Biol. Chem.*, **213,** 907 (1955).
2. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114,** 583 (1936).
3. Mackler, B., and Guest, G. M., *Proc. Soc. Exp. Biol. and Med.*, **83,** 327 (1953).
4. Harpur, R. P., and Quastel, J. H., *Nature*, **164,** 693 (1949).
5. Brown, D. H., *Biochim. et biophys. acta*, **7,** 487 (1951).
6. Hoare, D. S., and Kerly, M., *Biochem. J.*, **58,** 38 (1954).
7. Sols, A., and Crane, R. K., *Federation Proc.*, **12,** 271 (1953).
8. Brown, D. H., *J. Biol. Chem.*, **204,** 877 (1953).
9. Levine, R., Goldstein, M. S., Huddlestun, B., and Klein, S. P., *Am. J. Physiol.*, **163,** 70 (1950).
10. Wick, A. N., and Drury, D. R., *Am. J. Physiol.*, **173,** 229 (1953).

XUM

STUDIES ON THE MECHANISM OF ACTION OF 6-AZATHYMINE

I. BIOSYNTHESIS OF THE DEOXYRIBOSIDE

By WILLIAM H. PRUSOFF

(From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut)

(Received for publication, December 1, 1954)

Azathymine, the 6-nitrogen analogue of thymine, is a competitive antagonist of the growth of *Streptococcus faecalis* (8043) in a medium supplemented with thymine or its deoxyriboside, thymidine (1). Elion *et al.* (2) have observed that combinations of a purine analogue or a folic acid antagonist with azathymine produce an inhibitory effect on the growth of *S. faecalis* which is greater than the sum of the inhibitions caused by the individual compounds. Prusoff *et al.* (1) showed that azathymine not only interferes markedly with the utilization of *exogenous* thymine by this and several other microorganisms, but also of a related compound formed endogenously by *Lactobacillus leichmannii* in the presence of folic acid and vitamin B_{12}. Hakala *et al.* (3) have demonstrated that in the presence of Aminopterin, the outgrowth of a resistant mutant in a purine- and pyrimidine-free medium inoculated with a "wild strain" of *S. faecalis*, the rate of synthesis of thymine derivatives appears to be the limiting factor. Under these conditions azathymine causes rapid and irreversible cessation of reproductive activity. A possible parallelism to the situation which obtains in the development of resistance to Aminopterin or A-Methopterin in leucemia has been suggested (3).

Studies have now been made of a bacterial enzyme system (or systems) which is able to cleave thymidine and to transfer the deoxyribose moiety to azathymine. This deoxyriboside of azathymine (azathymidine) has possible applications as an inhibitor of the synthesis of deoxyribonucleic acid (DNA).

EXPERIMENTAL

Materials—Thymidine was generously supplied by L. Manson[1] and J. O. Lampen[2] of Western Reserve University. Thymidine also was isolated[3]

[1] Now a member of The Wistar Institute of Anatomy and Biology, University of Pennsylvania, Philadelphia, Pennsylvania.

[2] Now a member of The Squibb Institute for Medical Research, New Brunswick, New Jersey.

[3] In collaboration with W. L. Holmes.

from fresh calf thymus via the barium salt of thymidylic acid (4–7), which was dephosphorylated with moccasin venom phosphatase at pH 9.2 in 0.01 M $MgCl_2$ after the removal of barium by passage through a Dowex 50 column (H^+). Thymidine was purified by absorption on a Dowex 1 formate column at pH 10.5, followed by elution with 0.04 N HCOOH, and the eluate was lyophilized.

Azathymine was synthesized[4] by S. Greenbaum.

Preparation of Resting Cells—*S. faecalis* (8043) was grown in a basal medium (8) supplemented with thymine (2 mg. per liter) as described previously (9). In some instances cells were used which had been held in a frozen state, a treatment which did not alter nucleosidase activities.

Preparation of Cell-Free Extracts—2 volumes of phosphate buffer (0.5 M, pH 8.0) were added to 1 volume of *S. faecalis* cells and the suspension was placed for 1 hour in a sonic oscillator.[5] After centrifugation at 10,000 r.p.m.[6] the supernatant fraction either was used immediately, frozen, or lyophilized.

Methods of Analysis—Deoxyribose, thymine, and thymidine determinations were performed on perchloric acid extracts of the incubation mixtures by procedures described by Manson and Lampen (10), as modified by Hoffmann.[7] Ice-cold 25 per cent $HClO_4$ (0.1 volume) was added to the precooled incubation mixture. After 10 minutes the suspension was centrifuged at 0–5° and the supernatant fraction was decanted into 0.2 volume of 4 N sodium acetate. The method of Stumpf (11) was used for the determination of total deoxyribose; the free deoxyribose was determined by the procedure of Nelson (12).

Thymine and thymidine were determined spectrophotometrically in a Beckman spectrophotometer, model DU, or, if interfering substances were present, by microbial assay with either *Lactobacillus casei* (7469) (8) or *S. faecalis* (8). In the presence of thymine, thymidine was measured by the use of either *Leuconostoc citrovorum* (8081)[8] (14) or *Thermobacterium acidophilus* R26 (15).

Results

Studies with Resting S. faecalis Cells and Cell-Free Extracts—When washed cells (100 mg. wet weight) were incubated with thymidine (2

[4] We are grateful to G. H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, New York, for information concerning the method of synthesis.

[5] The magnetostriction oscillator employed was model R-22-3 (50 watts, 9 kc.) of the Raytheon Manufacturing Company.

[6] Type SS Servall centrifuge.

[7] Hoffmann, C. E., personal communication.

[8] In this paper the term *Leuconostoc citrovorum* (ATCC 8081) was chosen in keeping with common usage, although the name now applied is *Pediococcus cerevisiae* (13).

μmoles) in a phosphate buffer (0.05 M, pH 8.0), free thymine was formed. After an incubation of 15 minutes the alkaline absorption maximum shifted towards 290 mμ, with a corresponding increase in optical density at 300 mμ. Thymidine was cleaved completely within 4 hours, as indicated by the resultant absorption spectra in acid and alkali, which were identical to

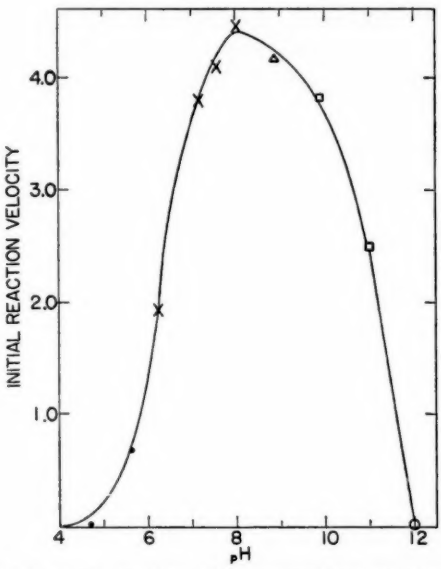


FIG. 1. The effect of pH on thymidine nucleosidase activity. The incubation mixtures contained thymidine (1 μmole), washed cells of *S. faecalis* (8043) (50 mg. wet weight), and the indicated buffers (final volume, 1.0 ml.). ●, 0.05 M acetate; ×, 0.05 M phosphate; △, 0.05 M glycylglycine; □, 0.05 M glycine; ○, 0.01 M NaOH. The preparation of the extract for deoxyribose analysis is described in the text. The initial reaction velocity is the decrement in Klett units (as measured by the procedure of Stumpf; Filter 50) divided by the initial 15 minute time interval.

those of free thymine. There was a corresponding disappearance of deoxyribose.

The rate of cleavage of thymidine by resting cells and cell-free extracts approached a maximum at about pH 8, as shown in Fig. 1. The initial reaction velocity was determined by measurement of the rate of deoxyribose disappearance during the initial 15 minute incubation period. Identical results were obtained when the rate of thymine formation was measured spectrophotometrically.

In Table I are presented the results of a comparison of the rate of cleav-

age of thymidine by freshly harvested and by frozen cells. The reaction velocities were identical for the time intervals measured and were constant for the initial 15 minute period. Freezing and subsequent thawing did not produce any detrimental effect on the nucleosidase activity of whole cells or cell-free extracts.

S. faecalis cells, which were grown in basal media (8) supplemented with either thymine, thymidine, or folic acid, showed no difference in the rate of cleavage of thymidine. However, when grown in a transfer broth composed of tryptone (1 per cent), peptonized milk (1 per cent), and tomato

TABLE I

Comparison of Rate of Thymidine Cleavage by Frozen and Non-Frozen S. faecalis Cells

Time	Reaction velocity	
	Non-frozen cells	Frozen cells
min.		
0– 5	3.6	3.4
5–10	3.0	3.2
10–15	3.6	3.6
15–20	2.8	2.8
20–30	1.8	1.4
30–60	1.0	0.9

The incubation mixtures contained thymidine (1 μmole), washed cells (50 mg. wet weight), and phosphate buffer (0.05 M, pH 8.0), in a total volume of 1.0 ml. After incubating at 37°, an extract was prepared for analysis by the procedure described in the text. The reaction velocity of thymidine cleavage was measured by the disappearance of deoxyribose as determined by the method of Stumpf. The reaction velocity is defined as the decrement in Klett units of Stumpf reaction (Filter 50) per minute. The reaction velocities were determined for the intervals of time indicated.

juice (20 per cent), a striking decrease in thymidine nucleosidase activity was observed. The factor (or factors) concerned with the appearance of high nucleosidase activity are being investigated.

As indicated in Fig. 2, 50 per cent of the thymidine was cleaved within 35 minutes by a first order reaction with a velocity constant of 0.023 per minute. Thus, under these conditions thymidine was cleaved at a rate of 2.3 per cent per minute. A maximal reaction velocity was attained at a concentration of 1 μmole of thymidine per ml., when the cell concentration was 50 mg. wet weight per ml. At higher levels of thymidine (5 μmoles per ml.), incomplete thymidine cleavage was observed, even after a prolonged incubation period.

Effect of Azathymine—The addition of azathymine to the incubation mixture resulted in an inhibition of deoxyribose disappearance. Fig. 3

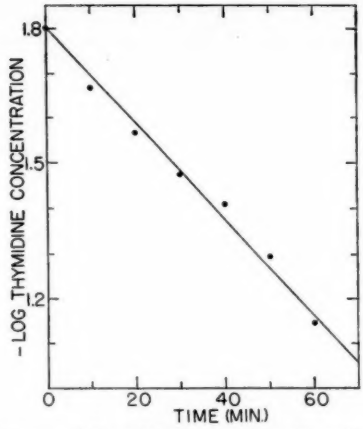


FIG. 2. Rate of cleavage of thymidine by *S. faecalis* cells. The composition of the incubation mixtures and the preparation of the extracts for analysis are described in the text.

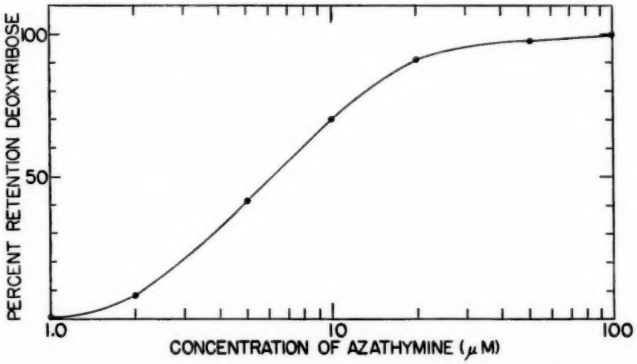


FIG. 3. The effect of azathymine on the disappearance of deoxyribose when cell-free extracts of *S. faecalis* were incubated with thymidine. The preparation of the cell-free extract is described in the text. The incubation mixtures were composed of thymidine (1 μmole), phosphate buffer (0.05 M, pH 8.0), cell-free extract (0.4 ml.), and varying concentrations of azathymine in a final volume of 1.0 ml. After incubation for 2 hours at 37°, an extract was prepared as described in the text and the deoxyribose content was determined.

shows that this inhibition was proportional to the concentration of azathymine. Since azathymine prevented the disappearance of deoxyribose during a prolonged period of incubation (Fig. 4), its effect cannot be attributed simply to a reduction in the rate of deoxyribose disappearance.

To determine whether the effect of azathymine was attributable to an interference with the cleavage of thymidine, to an inhibition of the dis-

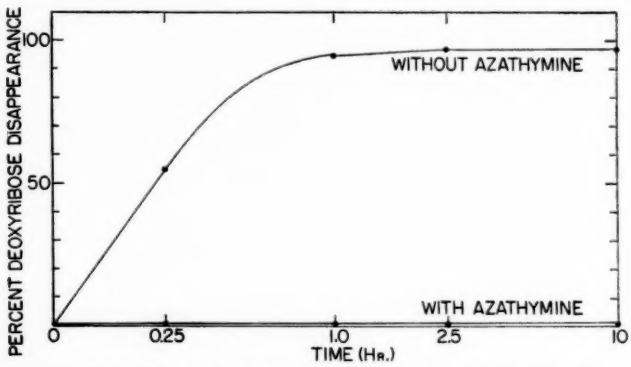


FIG. 4. The effect of time of incubation on the cleavage of thymidine and disappearance of deoxyribose in the presence and absence of azathymine.

TABLE II

Effect of Azathymine on Cleavage of Thymidine by Washed S. faecalis Cells

The incubation mixtures (1.0 ml.) contained *S. faecalis* cells (50 mg. wet weight), phosphate buffer (0.05 M, pH 8.1), plus the substrates listed in the table. After an incubation at 37° for the time intervals indicated, an extract was prepared and analyzed by the procedure described in the text.

Substrate	Incubation period	Deoxyribose		Thymidine		Thymidine + thymine	Thymine
		Total	Free	*L. citrovorum*	*T. acidophilus*	*S. faecalis*	Ultraviolet absorption
	hrs.	*μmoles*	*μmole*	*μmole*	*μmole*	*μmole*	*μmole*
Thymidine (1 μmole)	0	1.05	0.0	0.96	0.99	0.95	0.01
	0.25	0.53	0.0	0.36	0.42	0.97	0.57
	4.0	0.00	0.0	0.00	0.00	1.00	0.95
Thymidine (1 μmole) + azathymine (350 μmoles)	0	1.01	0.0	0.92	0.97		
	0.25	1.04	0.0	0.51	0.26		
	4.0	1.16	0.0	0.00	0.00		

appearance of the liberated "deoxyribose," or to a "binding" of the deoxyribose cleaved from thymidine, incubation mixtures (thymidine, buffer, cells), in the presence and absence of azathymine, were analyzed for thymine, thymidine, and free and total deoxyribose after 0.0, 0.25, and 4.0 hours of incubation. The results are presented in Table II. The disappearance of deoxyribose again was prevented by azathymine, but, since no

free sugar was released, the deoxyribose must have been held in a bound form. Microbial assays indicated that thymidine disappeared at essentially comparable rates whether in the presence or absence of azathymine. In the absence of azathymine, the rates of disappearance of thymidine and of deoxyribose were nearly identical. As the concentration of thymidine decreased, the relative amount of free thymine increased both in the presence and absence of azathymine. Thymine, produced by the degradation of thymidine, was not affected, since 100 per cent recovery of thymine was observed.

That *free* thymine was produced in the presence of azathymine was demonstrated in the following manner. Thymidine (4 μmoles), azathymine (1.4 mmoles), *S. faecalis* cells (200 mg.), and phosphate buffer (0.05 M, pH 8.0) in a total volume of 4.0 ml. were incubated for 0.0, 0.25, 2.5, and 10 hours. Perchloric acid extracts were prepared and diluted with 4 volumes of water. After adjustment of the pH to 8.5, the extracts were passed through a Dowex 1 acetate column (30 cm. × 1.3 cm.). Thymine and thymidine were washed from the column with phosphate buffer (0.1 M, pH 7.9); azathymine could be eluted subsequently by lowering the pH. The absorption spectra of the peak tube containing the eluates at pH 7.9, which were obtained from the incubation mixtures at the designated time intervals, were determined. Again, a shift to the right in the absorption maximum in alkali and an increase in the optical density at 300 mμ were observed. These changes are indicative of thymine formation. Thus, it is evident that the deoxyriboside of azathymine (azathymidine) had been formed.

Isolation of Deoxyriboside of Azathymine—Thymidine (71 mg.), azathymine (3.76 gm.), washed *S. faecalis* cells (8.8 gm. wet weight), and phosphate buffer (0.05 M, pH 8.0) in a final volume of 293 ml. were incubated in a water bath at 37° for 4 hours. After cooling, 0.1 volume of ice-cold 25 per cent $HClO_4$ was added and the formed precipitate was removed by filtration. The bulk of the $HClO_4$ in the filtrate was precipitated by neutralization with 6 N KOH and removed by filtration. The filtrate was lyophilized and the residue was extracted with 95 per cent EtOH. The alcohol was removed at reduced pressure and the residue was prepared for tritylation by drying over P_2O_5 *in vacuo* at 77° for 18 hours. Upon completion of the tritylation reaction (16), 4 volumes of 0.1 M $NaHCO_3$ were added and a white gelatinous precipitate was formed. After standing overnight the supernatant fluid was decanted and the residue was dissolved in EtOH and reprecipitated by the addition of 2 volumes of 0.1 M $NaHCO_3$. The crude tritylated derivative was filtered through a sintered glass funnel and the residual free azathymine was removed by washing with 10 ml. portions of 0.1 M $NaHCO_3$ until the optical density of the

filtrate reached a minimum. The crude trityl deoxyriboside of azathymine was detritylated by refluxing with glacial acetic acid in the manner described by Wolfrom *et al.* (17).

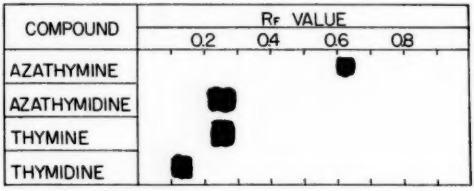


FIG. 5. Descending paper chromatogram of thymine, thymidine, azathymine, and azathymine deoxyriboside, with ethyl acetate saturated with phosphate buffer (0.05 M, pH 6.0) as the developing solvent. Occasionally the compounds would spread instead of forming distinct zones. Upon the suggestion of C. E. Carter, this problem was obviated by saturating the atmosphere with water in lieu of the solvent. The zones indicate ultraviolet-absorbing areas and were located by means of a Mineralight ultraviolet lamp, model SL-2537.

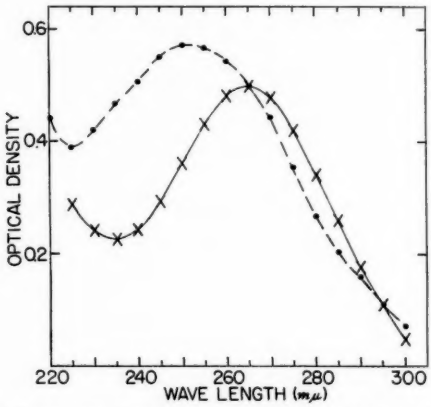


FIG. 6. Absorption spectra of azathymine deoxyriboside. ●, in 0.1 N HCl; ×, in 0.1 N NaOH.

Azathymine deoxyriboside was purified by ion exchange chromatography. The acidic solution of azathymidine was adjusted to pH 10.5 and absorbed on a Dowex 1 formate column (50 mm. × 24 mm.). After the column was washed with ammonium formate (0.01 M, pH 6.4), the azathymidine was eluted with formic acid (0.01 M) and the eluate was lyophilized. The molar ratio of azathymine to deoxyribose was 1.2. Paper chromatography of the compound resulted in only one spot (R_F 0.25)

and this contained deoxyribose (Fig. 5). The ultraviolet absorption spectra in acid and alkali are shown in Fig. 6. The absorption maximum was 265 mμ in acid and 252 mμ in alkali and the minimum was 236 mμ in acid and 226 mμ in alkali. Separation of azathymidine in gm. quantities has also been accomplished by counter-current techniques in lieu of tritylation, since the latter procedure occasionally resulted in poor yields. The problems encountered in this type of isolation of azathymidine, as well as some of its interesting biological properties, will be described elsewhere.

DISCUSSION

The presence of a highly potent nucleosidase in *S. faecalis*, which is capable of rapid cleavage of thymidine to free thymine, would suggest this mechanism as a possible pathway of thymidine utilization by the growing organism. However, it has been reported previously (1, 9) that azathymine or 5-bromouracil inhibited the growth of *S. faecalis* in a thymine-supplemented medium to a greater extent than in the presence of an equivalent molar concentration of thymidine. Thus, the pathway of thymidine utilization by this organism does not appear to be solely, if at all, via a preliminary cleavage with the formation of free thymine. Since the cleavage of thymidine in the presence of azathymine was not impaired and free sugar could not be detected, the formation of the deoxyriboside of azathymine appeared probable and was subsequently confirmed. Although the point of attachment of the deoxyribose moiety to the thymine analogue has not been established, as yet, it presumably is to nitrogen 2 of azathymine (3,5-dioxo-6-methyl-1,2,4-triazine), since this position corresponds to that now termed N-1 in thymine (2,4-dioxo-5-methylpyrimidine). When washed *S. faecalis* cells were incubated with thymine and the deoxyriboside of uracil, thymidine was formed; this proves that the enzyme can transfer the deoxyribose of a nucleoside to the N-1 position of a pyrimidine and offers presumptive evidence of an analogous constitution for azathymidine.

The mechanisms of transfer of the sugar moiety of a nucleoside to a purine or pyrimidine acceptor (18–22), as well as the enzymic synthesis of the deoxyriboside of compounds related to naturally occurring pyrimidines and purines (18, 23–27), have been described previously. Pyrimidine ribosides substituted in the 2 or 5 position also have been prepared and their biological activities investigated (23, 24, 28, 29). Lampen (27) and Kalckar (30) have reviewed the various mechanisms which are known to be involved in the cleavage of a nucleoside.

Preliminary observations[9] indicate that the deoxyriboside of azathymine is a more potent antagonist than azathymine of the growth of several

[9] Prusoff, W. H., unpublished experiment.

species of microorganisms (*S. faecalis*, *T. acidophilus* R26, *L. leichmannii* (ATCC 7469)). Thus, the first step of the mechanism of inhibition by azathymine may be the formation of its deoxyriboside. Whether the latter is incorporated into a polynucleotide such as DNA or a fragment of it is now under investigation. It is of interest to note that 8-azaguanine (31–36), 2-thiouracil (37), and 5-bromouracil (38–40) have been shown to be incorporated into nucleic acids. Hakala *et al.* (3) have presented evidence which suggests that azathymine may be incorporated into a unit more complex than the nucleoside.

Friedkin (25) has reported that azaguanine nucleosides synthesized by him, and assayed by Kidder for growth inhibition on *Tetrahymena* (*geleii*) *pyriformis*, had one-half the activity of free azaguanine on a molar basis. Kalckar (41) has postulated that azaguanine may exert its inhibitory activity by trapping the deoxyribose or ribose and in this manner may deprive the cell of essential building blocks. The inhibitory effect of

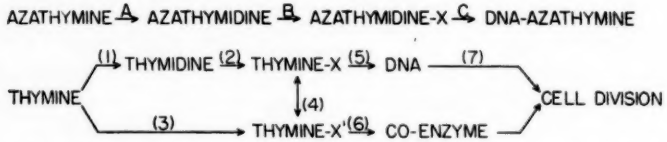


FIG. 7. Possible mechanisms of inhibition by azathymine and its deoxyriboside in relation to postulated pathways of thymine and thymidine utilization by *S. faecalis*.

azathymine cannot be accounted for by such a mechanism, since the deoxyriboside of azathymine has a greatly enhanced antimetabolic activity for several strains of microorganisms.[9]

Although azathymine may inhibit thymine utilization by *S. faecalis* at Reaction 1 or 3 (Fig. 7), the fact that azathymidine is a more potent antagonist than azathymine of the utilization of either thymidine or thymine suggests the possibility that azathymine is active only after deoxyribosidation (Reaction A). Part of the difficulty in attaining a satisfactory definitive interpretation of the data relates to the fact that the pathway of thymine utilization is not clear. Lampen and Jones (42) postulated that growth with thymine may involve a prior conversion to an intermediate, thymine-*X*; in the light of present day knowledge this compound could be the nucleoside or nucleotide. Postulated metabolic pathways of thymine metabolism are depicted in Fig. 7, in which thymine is converted to thymine-*X* or thymine-*X'*, either directly or via thymidine formation, prior to permitting cell division. In this scheme, thymine-*X* or -*X'* might be thymidine-5'-phosphate, a dinucleotide containing thymine, or a larger polynucleotide. Prior to their utilization for the synthesis of nucleic acids,

both orotic acid (43–45) and uracil (46, 47) appear to be converted to nucleotides. Although thymidylic acid, on a molar basis, has only about one-forty-fifth the activity of thymine or thymidine as a growth factor for *S. faecalis*, permeability factors may be involved.[9] Indeed, thymidylic acid, formed endogenously close to the site of utilization may be metabolized more efficiently than either thymine or thymidine. Accordingly, it is conceivable that both azathymine and azathymidine are converted to a derivative (Reaction B), *e.g.* the 5′-phosphate, before functioning efficiently, presumably by blocking synthesis of DNA (Reaction 4 or 5). If azathymine or azathymidine is incorporated into DNA (Reaction C), as would appear probable in view of observations with other analogues (31–40), it is possible that the abnormal DNA may prevent the normal reproduction of the cell (Reaction 7). Weaver and Shive (48) have postulated a catalytic rôle for thymidine; hence, the mechanism of azathymine inhibition may be at Reaction 6 (Fig. 7), *i.e.*, an inhibition of the formation of a thymidine-containing coenzyme.

Since thymidine (49, 50) is incorporated into the DNA of the rat and the chick embryo to a much greater extent than is thymine (51–53), an extension of these observations through a study of the effect of the deoxyriboside of azathymine on the development of various embryonic and neoplastic tissues is indicated.

SUMMARY

Washed cells or cell-free extracts of *Streptococcus faecalis* possess enzymes which cleave thymidine to free thymine with the disappearance of deoxyribose. Some of the properties of the nucleosidase are described. In the presence of 6-azathymine (3,5-dioxo-6-methyl-1,2,4-triazine) the disappearance of the deoxyribose of thymidine is prevented. The action of the analogue is accounted for by the formation of the deoxyriboside of azathymine and not by an inhibition of nucleosidase activity. The biosynthesis and isolation of the deoxyriboside of azathymine (azathymidine) are described, and the compound is shown to be a more potent inhibitor of the growth of several species of microorganisms than is azathymine. Possible mechanisms of inhibition of the growth of *S. faecalis* by azathymine and its deoxyriboside are discussed.

The author wishes to express his appreciation to Dr. A. D. Welch for advice concerning the preparation of the manuscript and for his interest in the development of this work, which is part of a continuing program on the study of pyrimidine and folic acid metabolism and their inhibition. Supported by grants from the American Cancer Society, as recommended by the Committee on Growth, National Research Council, and from the National Institutes of Health, Public Health Service.

XUM

BIBLIOGRAPHY

1. Prusoff, W. H., Holmes, W. L., and Welch, A. D., *Cancer Res.*, **14,** 570 (1954).
2. Elion, G. B., Singer, S., and Hitchings, G. H., *J. Biol. Chem.*, **208,** 477 (1954).
3. Hakala, M. T., Prusoff, W. H., and Welch, A. D., *Federation Proc.*, **13,** 223 (1954).
4. Simmons, N. S., Chavos, S. A., and Orbach, H. K., University of California at Los Angeles Atomic Energy project report No. 184 (1952).
5. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., *J. Am. Chem. Soc.*, **74,** 1724 (1952).
6. Volkin, E., Khym, J. X., and Cohn, W. E., *J. Am. Chem. Soc.*, **73,** 1533 (1951).
7. Hurst, R. O., Little, J. A., and Butler, G. C., *J. Biol. Chem.*, **188,** 705 (1951).
8. Lepper, H. A., Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 7th edition, 784 (1950).
9. Prusoff, W. H., *Proc. Soc. Exp. Biol. and Med.*, **85,** 564 (1954).
10. Manson, L. A., and Lampen, J. O., *J. Biol. Chem.*, **191,** 95 (1951).
11. Stumpf, P. K., *J. Biol. Chem.*, **169,** 367 (1947).
12. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
13. Felton, E. A., and Niven, C. F., *J. Bact.*, **65,** 482 (1953).
14. Sauberlich, H. E., *J. Biol. Chem.*, **181,** 467 (1949).
15. Hoff-Jørgensen, E., *Biochem. J.*, **50,** 400 (1952).
16. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **104,** 385 (1934).
17. Wolfrom, M. L., Burke, W. J., and Waisbrot, S. W., *J. Am. Chem. Soc.*, **61,** 1827 (1939).
18. MacNutt, W. S., *Biochem. J.*, **50,** 384 (1952).
19. Kalckar, H. M., *J. Biol. Chem.*, **158,** 723 (1945).
20. Friedkin, M., and Kalckar, H. M., *J. Biol. Chem.*, **184,** 437 (1950).
21. Manson, L. A., Thesis, Washington University (1949).
22. Manson, L. A., and Lampen, J. O., *J. Biol. Chem.*, **193,** 539 (1951).
23. Friedkin, M., and Roberts, D., *J. Biol. Chem.*, **207,** 257 (1954).
24. Strominger, D. B., and Friedkin, M., *J. Biol. Chem.*, **208,** 663 (1954).
25. Friedkin, M., *J. Biol. Chem.*, **209,** 295 (1954).
26. Prusoff, W. H., *Federation Proc.*, **12,** 358 (1953).
27. Lampen, J. O., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **2,** 363 (1952).
28. Visser, D. W., Lagerborg, D. L., and Pearson, H. E., *Proc. Soc. Exp. Biol. and Med.*, **79,** 571 (1952). Fukuhara, T. K., and Visser, D. W., *J. Biol. Chem.*, **190,** 95 (1951). Roberts, M., and Visser, D. W., *J. Biol. Chem.*, **194,** 695 (1952).
29. Prusoff, W. H., Holmes, W. L., and Welch, A. D., *Cancer Res.*, **13,** 221 (1953). Prusoff, W. H., *Proc. Soc. Exp. Biol. and Med.*, **85,** 567 (1954).
30. Kalckar, H. M., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 675 (1954).
31. Mitchell, J. H., Skipper, H. E., and Bennett, L. L., Jr., *Cancer Res.*, **10,** 647 (1950).
32. Heinrich, M. R., Dewey, V. C., Parks, R. E., Jr., and Kidder, G. W., *J. Biol. Chem.*, **197,** 199 (1952).
33. Matthews, R. E. F., *Nature*, **171,** 1065 (1953).
34. Matthews, R. E. F., *J. Gen. Microbiol.*, **10,** 521 (1954).
35. Bennett, L. L., Jr., Skipper, H. E., and Law, L. W., *Federation Proc.*, **12,** 300 (1953).
36. Mandel, H. G., Carló, P.-E., and Smith, P. K., *J. Biol. Chem.*, **206,** 181 (1954).
37. Jeener, R., and Rosseels, J., *Biochim. et biophys. acta*, **11,** 438 (1953).

38. Dunn, D. B., and Smith, J. D., *Nature*, **174,** 305 (1954).
39. Weygand, F., Wacker, A., and Dellweg, H., *Z. Naturforsch.*, **7b,** 19 (1952).
40. Zamenhof, S., and Griboff, G., *Nature*, **174,** 306 (1954).
41. Kalckar, H. M., *Biochim. et biophys. acta*, **12,** 250 (1953).
42. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170,** 133 (1947).
43. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195,** 257 (1952); **209,** 1 (1954).
44. Weed, L. L., *Cancer Res.*, **11,** 470 (1951).
45. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2027 (1954). Lieberman, I., Kornberg, A., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 2844 (1954).
46. Strominger, J. L., *Federation Proc.*, **12,** 277 (1953).
47. Rutman, R. J., Cantarow, A., and Paschkis, K. E., *Cancer Res.*, **14,** 119 (1954).
48. Weaver, J. M., and Shive, W., *J. Am. Chem. Soc.*, **75,** 4628 (1953).
49. Reichard, P., and Estborn, B., *J. Biol. Chem.*, **188,** 839 (1951).
50. Friedkin, M., Tilson, D., and Roberts, D., *Federation Proc.*, **13,** 214 (1954).
51. Brown, G. B., Roll, P. M., and Weinfeld, H., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **2,** 388 (1952).
52. Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153,** 203 (1944).
53. Holmes, W. L., Prusoff, W. H., and Welch, A. D., *J. Biol. Chem.*, **209,** 503 (1954).

THE METABOLISM OF *n*-BUTYRATE-C^{14} BY MAMMALIAN HEART MUSCLE*

By VICTOR LORBER† and MARGARET COOK

(From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio, and the Department of Physiology, School of Medicine, University of Minnesota, Minneapolis, Minnesota)

(Received for publication, December 18, 1954)

The anomalous increase in cardiac glycogen during ketosis and the induction of a similar change by the infusion of ketone bodies or butyrate (4) suggest that a pathway allowing for the net accumulation of carbohydrate from fat may exist in heart. In an effort to assess this possibility, the conversion of *n*-butyrate-3-C^{14} to acetoacetate, β-hydroxybutyrate, glycogen, lactate, pyruvate, and fumarate was studied in the isolated blood-perfused or sliced mammalian heart. Incidental observations on the conversion of butyrate to crotonate and vinyl acetate were also made.

Methods and Materials

The isolated cat heart was perfused with heparinized cat blood by the Langendorff method with a modification of a system previously described (5). Rabbit ventricle was sliced free-hand or with the Stadie-Riggs microtome (6) or minced with a stainless steel tissue press (7). The tissue was suspended in Krebs-Ringer-phosphate solution (8), 7.5 to 10 ml. per gm. of wet tissue, and incubated with shaking at 37.5° in oxygen for approximately 90 minutes. When desired, respiratory CO_2 was collected in an external absorber containing carbonate-free NaOH.

Experiments were carried out with sodium butyrate-3-C^{14} as added substrate either alone or in addition to acetoacetate, pyruvate, crotonate, or vinyl acetate in various combinations. At the end of the perfusion or incubation, compounds of interest were prepared for chromatography, with or without the addition of carrier, essentially as described previously (9), and separated by partition chromatography, as illustrated in Fig. 1. Butyrate, crotonate, and vinyl acetate were separated on a buffered Celite column (10) (see Fig. 2). While the resolution of these three acids deter-

* This work was supported in part by grants from the American Heart Association and from the Graduate School of the University of Minnesota. The radiocarbon used was obtained on allocation from the United States Atomic Energy Commission. Preliminary reports of this work have appeared (1–3).

† Career Investigator of the American Heart Association. Present address, Department of Physiology, University of Minnesota, School of Medicine, Minneapolis 14, Minnesota.

XUM

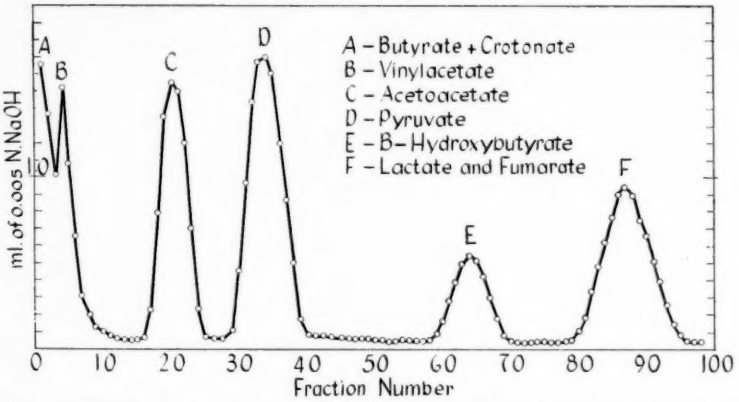


FIG. 1. The stationary phase consists of 14 gm. of Johns-Manville Celite (No. 535) thoroughly mixed with 7 ml. of 3 N H_2SO_4 and firmly packed in a tube of 22 mm. inner diameter. 90 ml. of benzene, 250 ml. of 1 per cent, 200 ml. of 2 per cent, 350 ml. of 3 per cent, and 150 ml. of 5 per cent *n*-butanol in $CHCl_3$ are put through the column in the order listed. All solvents are equilibrated with 3 N H_2SO_4. 10 ml. fractions are collected. Fumarate and lactate, which are eluted as a single fraction, are separated on another column with 0.2 N H_2SO_4 as the stationary phase and 5 per cent *n*-butanol in $CHCl_3$ as the solvent.

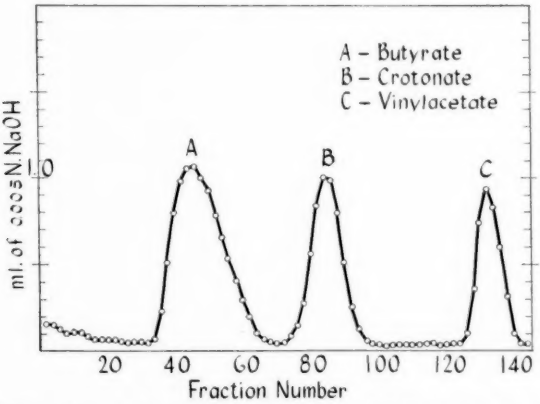


FIG. 2. The stationary phase consists of 18 gm. of Celite and 9 ml. of 2 M potassium phosphate buffer, pH 7.2. 700 ml. of 1.5 per cent, 300 ml. of 5 per cent, and 500 ml. of 20 per cent *n*-butanol in $CHCl_3$ are put through the column in the order listed. All solvents are equilibrated with the buffer. 10 ml. fractions are collected. Only the titration values for the even-numbered fractions are plotted.

mined by titration appears to be satisfactory by this method, there is actually extensive trailing as determined by radioassay, and because of the high level of radioactivity in the butyrate it was necessary to pass the crotonate and vinyl acetate through the column a total of three times to remove the last traces of butyrate.

Acetoacetate was decarboxylated by heat or aniline citrate (11, 12), with good agreement between the two methods (Table I). The resulting acetone was isolated as the mercury complex (11), distilled from 2 N HCl, and reprecipitated as the mercury complex. An aliquot was then converted to CO_2 (13) for radioassay, and free acetone was regenerated from the

TABLE I

Comparison of Degradation of Various Compounds by Different Procedures

Compound	Method of degradation	Specific activity*			
		COOH	C-2	C-3	C-4
Acetoacetate	Van Slyke	695	0	2577	0
	Aniline citrate	694			
β-Hydroxybutyrate	Van Slyke	326		822†	
	Via crotonate, butyrate, and azide	341		858†	
Lactate	$KMnO_4$ and NaOI	68	0	0	
	Via guanidine salt	68	0	0	
Pyruvate	Yeast and NaOI	8	0	0	
	Ceric sulfate and azide	11	468		

* Specific activity, unless otherwise indicated, is expressed as counts per minute per mg. of carbon.

† Specific activity of acetone carbon × 3.

remainder for further degradation by the iodoform reaction (14). β-Hydroxybutyrate was degraded by a modification of the method of Van Slyke (11), and the mercury-acetone complex was further degraded as described above. By this method, the yields of carboxyl carbon are 10 to 15 per cent high and the acetone correspondingly low. The results obtained in this way are checked in the following manner. The guanidine salt (15) of β-hydroxybutyrate was prepared (m.p., 181–182°, uncorrected), the free acid regenerated by passage through a Dowex 50 column, and an aliquot converted to crotonate by a constant volume steam distillation from 50 per cent H_2SO_4. The crotonate was reduced to butyrate in the presence of zinc and phosphoric acid (16), isolated on a buffered Celite column, and decarboxylated by azide (17). The resulting propylamine was isolated by alkaline distillation and converted to CO_2 (13). Another aliquot of the

free β-hydroxybutyrate was degraded in the usual way. It can be seen in Table I that the specific activities of the fractions derived by the different methods are in good agreement. The azide degradation has been checked with highly active butyrate-3-C^{14} and found to be reliable. The agreement with the Van Slyke procedure, in spite of the imperfect stoichiometry in the latter method, may be fortuitous and result from the labeling pattern found in the present experiments. Since the average specific activity of carbons 2 to 4 is not very different from that of the carboxyl group, a small contamination of the latter by the former will not greatly alter the observed specific activity.

Lactate was degraded as described previously (18). In one experiment the guanidine salt (15) was prepared from an aliquot (m.p., 163.5–164.5°, uncorrected; mixed melting point not depressed), the free acid regenerated by passage through a Dowex 50 column, and the degradation repeated with good agreement (Table I). Pyruvate was decarboxylated by ceric sulfate (19) and the resulting acetate further degraded by azide (20) or counted directly as the barium salt. In one experiment an aliquot was also decarboxylated by incubation with a washed suspension of dried brewer's yeast (Anheuser-Busch) and the resulting acetaldehyde further degraded with NaOI (18). The widely divergent results (Table I) obtained by these two methods will be considered later. Fumarate, isolated in one experiment by chromatography and sublimation, was decarboxylated with *Lactobacillus arabinosus* to yield lactate and CO_2 (21, 22), and the lactate was then further degraded (18). The culture of *L. arabinosus* was a gift of Dr. J. S. Lee.

Crotonate and vinyl acetate were reduced to butyrate (16), and the butyrate was isolated chromatographically. Butyrate was decarboxylated by azide (17) and the resulting propylamine either isolated by alkaline distillation and converted to CO_2 (13) or further oxidized to propionate (17), isolated on a 0.5 N acid Celite column developed with 1 per cent *n*-butanol in chloroform, and then converted to CO_2 (13). Radioassay was carried out as described previously (9). Glycogen was isolated by the method of Stetten and Boxer (23).

Sodium butyrate-3-C^{14} was kindly prepared by Mr. S. Patel from ethyl iodide-1-C^{14} by the malonic ester synthesis and separated from non-isotopic acetate on a neutral column of Celite by use of 1 per cent *n*-butanol in chloroform to elute the butyrate. A sample of vinyl acetate was kindly furnished by Dr. H. A. Barker. All other compounds except lithium lactate (24) and sodium acetoacetate (12) were commercial reagents. Johns-Manville Celite (No. 535) was used throughout. Lactate was determined by the method of Barker and Summerson (25), pyruvate according to Friedemann and Haugen (26) or Fromageot and Desnuelle (19), and acetoacetate by the method of Rosenthal (27) or of Krebs and Eggleston (12).

RESULTS AND DISCUSSION

In Table II, data are presented on the rate of butyrate oxidation by the perfused heart and by ventricular slices. The total metabolism of the latter preparation is only about one-fifth of the former, but butyrate oxidation is depressed by only one-half, even at the much lower butyrate concentration employed. This, together with the greater contribution to the total CO_2 made by butyrate, indicates a lesser dilution with endogenous substrate in the slice experiments, as might be expected. While these limited observations do not allow a critical comparison of the performance

TABLE II

Oxidation of Na Butyrate-3-C^{14} by Perfused Heart versus Sliced Ventricle

Experiment No.*	Preparation	Labeled substrate concentration and total activity	Duration	CO_2 output	Total activity in CO_2	Butyrate oxidized to CO_2	Per cent total CO_2 from added butyrate
			min.	*μmoles per gm. per hr.*	*c.p.m.*	*μmoles per gm. per hr.*	
1	Perfused cat heart, 12.5 gm., 100 ml. blood volume	0.01 M, 1.71×10^6 c.p.m.	90	149	34,360	1.07†	2.87‡
2	Rabbit ventricle slices, 6 gm., 60 ml. Krebs-Ringer	0.002 M, 2.98×10^5 c.p.m.	80	28	11,170	0.56	8.0

* A given experiment in this and in Tables III and IV bears the same number.

† $\frac{34{,}360}{1.71 \times 10^6} \times \frac{1000\ \mu\text{moles (total butyrate)}}{12.5\ \text{gm.}} \times \frac{6}{9}\ \text{hour} = 1.07\ \mu\text{moles per gm. per hour.}$

‡ $\frac{1.07 \times 4}{149} \times 100 = 2.87$ per cent. It is assumed that the 4 carbons of butyrate are converted to CO_2 at an approximately equal rate.

of the two types of preparation, they indicate that the slice offers the advantage of a reduced isotope dilution and consequently a more efficient utilization of isotopic substrate. The added advantage of simplicity and the fact that the isotope distribution pattern in acetoacetate isolated from the two preparations is the same (Table III) prevailed in determining the choice of technique employed in the major portion of the present work.

In Table III, the isotope distribution patterns are presented for acetoacetate, β-hydroxybutyrate, crotonate, and vinyl acetate. These patterns may be regarded as qualitatively similar to those existing in the actual intermediates of butyrate oxidation, the coenzyme A derivatives, from which the free acids are liberated by the deacylase activity of the tissues. It is of interest that the present results are consistent with the reaction

TABLE III

Distribution of Isotope in Products of Butyrate Oxidation

Experiment No.	Preparation	Additions (final concentration)	Specific activity* of carbon atoms											
			Acetoacetate				β-Hydroxybutyrate				Crotonate		Vinyl acetate	
			$CH_3-CO-CH_2-COO^-$				$CH_3-CHOH-CH_2-COO^-$				$CH_3-CH=CH-COO^-$		$CH_2=CH-CH_2-COO^-$	
1	Perfused cat heart	0.01 M Na butyrate-3-C^{14} (1.71×10^3 counts per μmole), 0.01 M Na acetoacetate	0	720 (2,440)	0	274 (926)								
2	Rabbit ventricle slices	0.002 M Na butyrate-3-C^{14} (2.48×10^3 counts per μmole), 0.002 M Na acetoacetate	0	1,053 (3,390)	0	305 (981)	0	591 (12,800)	0	160 (3,460)				
3	" "	0.0017 M Na butyrate-3-C^{14} (2.66×10^4 counts per μmole), 0.0013 M Na acetoacetate, 0.00033 M Na pyruvate	0	2,577 (70,800)	0	695 (19,100)	0	1,281 (274,000)	0	342 (73,200)				
4	" "	" "						858		341				
5	" "	0.002 M Na butyrate-3-C^{14} (2.66×10^4 counts per μmole)		720†				615		224				
								2190†			66	4		
											210†		5†	

6	Rabbit ventricle mince	0.0011 M Na butyrate-3-C^{14} (2.66×10^4 counts per μmole), 0.00057 M Na crotonate, Na vinyl acetate			618 (9210)	2 (30)	75 (834)	0

* The figures in parentheses in this and Table IV are the observed values corrected for dilution due to the addition of carrier at various stages of isolation.

† Total counts recovered in the compound.

sequence postulated for butyrate oxidation on the basis of enzymatic studies (28).

A very approximate measure of the extent to which the various free acids may accumulate in heart tissue during butyrate oxidation in the absence of added carrier is provided by the results in Experiments 4 and 5 (Table III). In Experiment 5, the total activity was determined in the chromatographic fractions containing acetoacetate and β-hydroxybutyrate. This was found to be equivalent to 0.027 μmole (720/26,600) and 0.082 μmole (2190/26,600) of butyrate, respectively, and corresponds to about 10 per cent of the butyrate converted to CO_2 in a comparable period (based on the CO_2 values in Experiment 2, Table II). In the case of the unsaturated acids, a rough

TABLE IV

Distribution of Isotope in Carbohydrate Precursors

Experiment No.	Specific activity of carbon atoms								
	Lactate			Pyruvate			Fumarate		Glycogen
	CH_3 —	$CHOH$ —	COO^-	CH_3 —	CO —	COO^-	$=CH$ —	COO^-	
1					73	18			0
					(1050)	(251)			
2	0	0	45		30	67			*
			(170)		(1990)	(1580)			
3	0	0	68	0	0	8			*
			(2380)			(4600)			
4	0	7	382				0	218†	

* Glycogen was not recovered in detectable amounts in these experiments.

† Average of the specific activities of the two carboxyl groups (230 and 206) obtained by degradation with *L. arabinosus*.

indication of the extent of accumulation in the absence of added carrier is given by the total activities recovered in crotonate and vinyl acetate from Experiments 4 and 5 combined (Table III). In the case of crotonate, this is equivalent to approximately 0.008 μmole of butyrate (210/26,600) in the combined experiment. While the losses in handling prior to chromatography were probably quite large in these experiments, even if recoveries were as low as 50 per cent, which seems unlikely, total crotonate accumulation remains trivial.

In Table IV are presented the observations on glycogen and some of its precursors. From the perfused heart (Experiment 1) appreciable glycogen was isolated, but it contained no activity. In the two slice experiments (Nos. 2 and 3), in which an effort was made to isolate glycogen, none was found. It is not clear at present whether the failure to label the glycogen in Experiment 1 represents a failure of glycogen synthesis under the condi-

tions employed or a limited ability in heart to form glycogen from lactate or pyruvate. The results with lactate indicate that this compound is largely if not entirely labeled in the carboxyl group. In Experiment 4, in which the α-carbon contains about 2 per cent of the activity noted in the carboxyl group, the lactate was not characterized as the guanidine salt. The finding that fumarate isolated in the same experiment is only labeled in the carboxyl carbons is consistent with a similar distribution pattern in the lactate (29) and suggests that the activity noted in the α-carbon of lactate in this experiment may have been due to a contaminant.

In Experiments 1 and 2 (Table IV), it is noted that the distribution of isotope in pyruvate is clearly inconsistent with the pattern observed in lactate and fumarate. Degradation of the pyruvate in these two experiments was carried out by chemical means. In Experiment 3 the more specific yeast carboxylase was employed, with the result that detectable activity is noted only in the carboxyl group. The low observed specific activity results from the fact that carrier pyruvate was added on the basis of the total activity found in the pyruvate isolated chromatographically. It has since been established that the major fraction of this activity and the aberrant results in Experiments 1 and 2 are due to a compound that migrates with pyruvate on the Celite column. The nature of this compound is under investigation.

On the basis of the present results, the question of a possible net conversion of fat (via ketone bodies) to glycogen in heart can be answered tentatively in the negative. Starting with C^{14}-butyrate or acetoacetate labeled as in the present experiments, suggested intermediary pathways (30, 31) that might account for such a transformation require an appreciable isotope distribution in the non-carboxyl positions of such carbohydrate precursors as lactate, pyruvate, and fumarate. The conventional schemes, with which the present results are in accord (32), are inconsistent with a net formation of carbohydrate from fat. The possibilities exist that an alternative pathway may operate under the conditions of ketosis *in vivo*, or may be such as to lead to conventional isotope distribution patterns. Alternatively, the anomalous increase in cardiac glycogen that occurs in ketosis may be interpreted as a carbohydrate-sparing effect which results from the high rate of oxidative metabolism of acetoacetate by heart tissue (12, 33).

SUMMARY

Acetoacetate, β-hydroxybutyrate, crotonate, vinyl acetate, lactate, pyruvate, and fumarate have been isolated from the perfused, sliced, or minced mammalian heart metabolizing *n*-butyrate-3-C^{14}, and their isotope distribution patterns determined. The results are consistent with the reaction sequence postulated for butyrate oxidation on the basis of enzymatic

studies (28) and with the reactions of the Krebs cycle as the main route of interconversion of the products of butyrate oxidation to carbohydrate precursors (32). In the absence of added carrier, acetoacetate and β-hydroxybutyrate have been found to accumulate to a slight extent during butyrate oxidation in the heart.

BIBLIOGRAPHY

1. Lorber, V., *Federation Proc.*, **11,** 97 (1952).
2. Cook, M., *Federation Proc.*, **13,** 29 (1954).
3. Lorber, V., *Federation Proc.*, **13,** 91 (1954).
4. Lackey, R. W., Bunde, C. A., and Harris, L. C., *Proc. Soc. Exp. Biol. and Med.*, **66,** 433 (1947).
5. Lorber, V., *Circulation Res.*, **1,** 298 (1953).
6. Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, **154,** 687 (1944).
7. Bittner, J. J., and Imagawa, D. T., *Cancer Res.*, **10,** 739 (1950).
8. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis, 119 (1949).
9. Lorber, V., Utter, M. F., Rudney, H., and Cook, M., *J. Biol. Chem.*, **185,** 689 (1950).
10. Bueding, E., and Yale, H. W., *J. Biol. Chem.*, **193,** 411 (1951).
11. Van Slyke, D. D., *J. Biol. Chem.*, **32,** 455 (1917).
12. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **39,** 408 (1945).
13. Van Slyke, D. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.*, **191,** 299 (1951).
14. Ardagh, E. G. R., Barbour, A. D., McClellan, G. E., and McBride, E. W., *J. Ind. and Eng. Chem.*, **16,** 1133 (1924).
15. Packham, M. A., and Butler, G. C., *J. Biol. Chem.*, **194,** 349 (1952).
16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis, 172 (1949).
17. Mosbach, E. H., Phares, E. F., and Carson, S. F., *Arch. Biochem. and Biophys.*, **33,** 179 (1951).
18. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159,** 475 (1945).
19. Fromageot, C., and Desnuelle, P., *Biochem. Z.*, **279,** 174 (1935).
20. Phares, E. F., *Arch. Biochem. and Biophys.*, **33,** 173 (1951).
21. Lee, J. S., and Lifson, N., *J. Biol. Chem.*, **193,** 253 (1951).
22. Korkes, S., and Ochoa, S., *J. Biol. Chem.*, **176,** 463 (1948).
23. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155,** 231 (1944).
24. Friedemann, T. E., and Graeser, J. B., *J. Biol. Chem.*, **100,** 291 (1933).
25. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138,** 535 (1941).
26. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943).
27. Rosenthal, S. M., *J. Biol. Chem.*, **179,** 1235 (1949).
28. Mahler, H. R., *Federation Proc.*, **12,** 694 (1953).
29. Lorber, V., Lifson, N., Wood, H. G., Sakami, W., and Shreeve, W. W., *J. Biol. Chem.*, **183,** 517 (1950).
30. Sakami, W., and Lafaye, J. M., *J. Biol. Chem.*, **193,** 199 (1951).
31. Henze, M., and Müller, R., *Z. physiol. Chem.*, **193,** 88 (1930).
32. Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, **176,** 1263 (1948).
33. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **42,** 294 (1948).

XUM

REACTIONS OF CYTOCHROMES *a* AND a_3*

I. STUDIES OF OXIDATION AND REDUCTION OF THE PIGMENTS IN A PURIFIED PREPARATION

By LUCILE SMITH

(From the Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, August 16, 1954)

The observations of Keilin and Hartree (1) on the reactions of the heart muscle cytochrome system indicated that at least four components can be distinguished. These were named cytochromes *a*, a_3, *b*, and *c*. They showed that the bands in the absorption spectrum of reduced cytochromes *a* and a_3 overlap at 605 and 448 mμ, but that the two components could be distinguished by observing the changes in the absorption bands in the presence of respiratory inhibitors such as carbon monoxide and cyanide. Only a small part of the absorption band at 605 mμ changes on addition of CO or CN^-, while most of the band at 448 mμ shifts on addition of these substances. It was concluded that most of the absorption band at 605 mμ is due to cytochrome *a* which does not combine with CO or CN^-, while cytochrome a_3 is responsible for the largest part of the band at 448 mμ. Keilin and Hartree pointed out that cytochrome a_3 showed many properties of the heart muscle oxidase, and recently Chance (2) has studied the photochemical decomposition of the carbon monoxide compound of heart muscle cytochrome a_3. Chance also showed (3) by kinetic studies that cytochromes *a* and a_3 in heart muscle extract are closely related in activity. In spite of the difference in reaction with carbon monoxide and cyanide observed at the two wave-lengths corresponding to reduced cytochromes *a* and a_3, it has sometimes been assumed (4, 5) that the absorption bands at 605 and 445 mμ are those of only one pigment.

A preparation from heart muscle containing cytochromes *a* and a_3 and showing strong cytochrome *c* oxidase activity has been described (6). Since the preparation is optically clear, it can be used for spectrophotometric studies of the reactions of cytochromes *a* and a_3. This paper describes two additional purification steps in the preparation as well as some observations on the oxidation and reduction of the pigments.

Methods

Preparation of Cytochromes a + a_3 from Heart Muscle—The preparation was made as described previously (6), with the following two modifications.

* This research was supported by a grant from the Division of Grants and Fellowships, National Institutes of Health, United States Public Health Service.

(*a*) The phosphate extract of heart muscle obtained by centrifugation of the homogenate from the Waring blendor is precipitated by lowering the pH of the cold mixture (below 5°) to 5.6 by addition of cold 2 M acetic acid. The precipitate collected by centrifugation at 2500 × *g* is washed once in the centrifuge with cold distilled water, then suspended in 0.1 M phosphate buffer, pH 7.4, up to a final volume of 300 ml.; a hand-operated ground glass homogenizer is used to disperse the clumps if necessary. Sodium cholate is then added to make the mixture 0.8 per cent and the preparation is digested with trypsin (0.25 mg. per ml.) and fractionated with ammonium sulfate as described previously. The Type I preparation is made up to a final volume of about 30 ml. From this the Type II preparation is prepared by diluting the preparation four to five times with 0.1 M buffer and centrifuging as described.

The acid precipitation serves to increase the purity (decrease the dry weight) of the final preparation somewhat, but does not remove the final trace of pigment with a peak in the absorption spectrum around 560 mμ. Thus the acid precipitation is not necessary if the preparation is only to be used as a strong cytochrome *c* oxidase or as an optically clear preparation of cytochromes $a + a_3$.

(*b*) The trace of pigment with an absorption peak at about 560 mμ, which is reduced by $Na_2S_2O_4$ but not by cytochrome *c* plus ascorbate, can be removed from the Type II preparation by adsorption of the cytochromes $a + a_3$ on calcium phosphate gel. The gel is prepared according to the directions of Keilin and Hartree (7) and used as a suspension containing 9 gm. per 100 ml. A test series is run with different amounts of gel added to a series of 4 ml. portions of the enzyme preparation. After standing for about 15 minutes, the samples of gel are collected by centrifugation, then stirred with 6 ml. portions of a mixture of 13 per cent ammonium sulfate + 0.5 per cent sodium cholate to elute the pigments. The gel is then removed by centrifugation. It was often found that the ratio of 3 ml. of gel suspension to 4 ml. of enzyme preparation was satisfactory to adsorb most of the cytochromes *a* and a_3 and none or very little of the contaminating pigment. This procedure results in very little increase in cytochrome *c* oxidase activity expressed on a dry weight basis.

Heart muscle succinic oxidase was prepared by a slight modification of the usual Keilin and Hartree method (8).

Measurement of Cytochrome c Oxidase Activity—Cytochrome *c* oxidase activity was determined by measuring the rate of oxidation of reduced cytochrome *c* at 20° or 25°. The change in optical density at 550 mμ was recorded automatically on the chart of a recording spectrophotometer (9) after addition of 0.1 ml. of the diluted enzyme (diluted at least 100

times in cold distilled water) to reduced cytochrome *c* in phosphate buffer. The final concentration of buffer in the reaction mixture was 0.067 M, pH 7.0. The cytochrome *c* was reduced by hydrogen and palladium, as described previously (10), and in these experiments the concentration of cytochrome *c* was around 15 μM.[1] The cytochrome *c* oxidase activity is expressed as the velocity constant for the reaction, which is first order with respect to the concentration of reduced cytochrome *c*.

Measurement of Absorption Spectra—The absorption spectra of the preparations in the oxidized or reduced forms or after addition of a number of reagents were obtained with the automatic recording spectrophotometer, with buffer in the reference cuvette. In some experiments, difference spectra were recorded by placing the preparation in the oxidized form in one cuvette and the reduced pigment in the other, or by adding reagents to one of the cuvettes with either the oxidized or reduced pigment in the blank cuvette. To obtain spectrophotometric data, the preparation was usually diluted in either 1 per cent sodium cholate or a mixture of 13 per cent ammonium sulfate + 0.5 per cent cholate, since, as discussed previously (6), only highly diluted preparations are stable in the absence of cholate or ammonium sulfate. In several experiments, dilute preparations in phosphate buffer (0.1 M, pH 7.0) were examined. When the preparation was diluted between 10- to 20-fold with buffer, the experiments were done as rapidly as possible, since at this dilution turbidity will usually begin to develop after a short time.

Results

Activity of Preparation in Relation to Cytochrome Content—The extent of purification of the preparation (expressed as cytochrome *c* oxidase activity divided by the dry weight) is difficult to assess, since, as will be discussed in a subsequent paper, the velocity constant for the oxidation of reduced cytochrome *c* is observed to vary with the total concentration of cytochrome *c* in the test mixture. When both heart muscle extract and the purified preparation are assayed in the presence of 15 μM cytochrome *c*, about a 20-fold purification is usually observed in the preparation. At lower concentrations of cytochrome *c* the measured purification is several times greater, since the concentration of cytochrome *c* has less effect on the oxidase in the heart muscle preparation than on the purified oxidase. The calculated Q_{O_2} of the purified preparation at 25° and 15 μM cytochrome *c* usually varies from about 5000 to 10,000.

The absorption spectrum of the reduced preparation, shown in Fig. 1, has peaks only at 605 and 445 mμ. The ratio of the two peaks was found to be constant in a number of different preparations, as shown in the data

[1] μM represents micromolar (micromoles per liter) concentration.

of Table I, and in freshly made preparations the ratio of the cytochrome *c* oxidase activity to either peak is fairly constant. When the preparation stands at 4° for several weeks, there is observed a slow decrease in the cytochrome *c* oxidase activity, but the absorption spectrum of the preparation reduced with $Na_2S_2O_4$ remains unchanged.

The quotient of the cytochrome *c* oxidase activity divided by the height of the absorption peak at 605 mμ may be compared with similar values obtained with heart muscle extract. For this comparison the difference in optical density between the oxidized and reduced forms at 605 mμ is used,

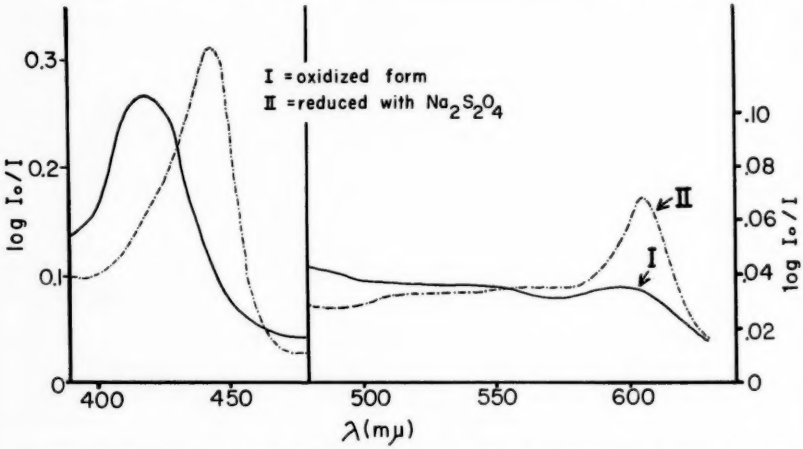


FIG. 1. Spectra of the purified cytochrome *c* oxidase preparation obtained by use of the recording spectrophotometer. The preparation was diluted in 1 per cent sodium cholate.

since this is the only value that can be obtained for the turbid heart muscle extracts (3). The observed values for cytochrome *c* oxidase activity (velocity constant at 25° × dilution) divided by the increase in optical density on reduction at 605 mμ varied from 168 to 199. These values were obtained with a cytochrome *c* concentration of 15 μM. A group of active heart muscle extracts assayed similarly gave values from 188 to 230. Thus the original activity of the enzyme on the heart muscle particles seems to be retained.

Effect of Sodium Cholate on Cytochrome c Oxidase Activity—The effect of the concentration of sodium cholate present in the reaction mixture on the cytochrome *c* oxidase activity of a purified preparation and some heart muscle extract is presented in Fig. 2. In the assay method used for cytochrome *c* oxidase activity, the purified preparation is diluted at least 3000

times in the reaction mixture and the cholate concentration is less than 0.00017 per cent. When the spectrophotometric investigations are made in the presence of 1 per cent cholate, the oxidase activity is largely inhibited.

The effect of cholate on the enzyme on heart muscle particles is less than that on the purified preparation. It was also found that, if strong cholate solution is added to a concentrated heart muscle extract to make a cholate concentration of 3 per cent and the extract is then diluted to run the assay, a stimulation of the oxidase activity results, whereas an inhibition is observed if the same final concentration is present in the assay mixture before addition of the enzyme.

TABLE I

Ratio of Peaks at 445 and 605 mμ in Number of Different Preparations Reduced with $Na_2S_2O_4$

Preparation No.	$D_{445\ m\mu}$	$D_{605\ m\mu}$	$\frac{D_{445}}{D_{605}}$
1	5.20	1.18	4.4
2	5.45	1.18	4.6
3	4.09	0.92	4.4
4	7.28	1.52	4.8
5	6.90	1.60	4.3
6	4.28	1.90	4.4
7	3.68	0.86	4.3
8	4.80	1.08	4.4
9	2.67	0.62	4.3
Average.....			4.4

The optical density measurements at the two wave-lengths were made in a Beckman spectrophotometer with a number of different preparations diluted with a mixture of 0.5 per cent cholate and 13 per cent ammonium sulfate. The figures represent the observed optical densities multiplied by the dilution. The preparations were made with a number of variations of the usual procedure.

Reduction and Oxidation of Cytochromes a and a_3—The cytochromes *a* and a_3 of the purified preparation can be reduced by a number of reagents. In the first studies to be described, the preparation was diluted for the spectrophotometric measurements with 1 per cent sodium cholate in 0.1 M phosphate buffer neutralized to pH 7.4. To show the spectral changes more clearly, difference spectra were plotted automatically with the recording spectrophotometer. A base-line was first obtained with oxidized preparation in both cuvettes; then the reagents were added to one cuvette and the difference spectrum was recorded.

When cytochrome *c* (final concentration around 2 to 3 μM) plus ascorbic

acid (0.02 M) is added to the preparation diluted about 10-fold in 1 per cent cholate, a slow oxygen uptake ensues, so that the oxygen concentration in the solution reaches zero in about 30 minutes. With a combination of cytochrome *c* and *p*-phenylenediamine (0.0005 M) the oxygen uptake is faster and the oxygen in solution is exhausted in 7 to 10 minutes. The recording instrument used can scan the spectrum from 650 to 370 mμ in about 30 seconds, and more rapid recordings can be made of the extent of reduction of cytochromes *a*, a_3, and *c* during the aerobic steady state than after the oxygen has been exhausted. These are shown in Figs. 3 and 4.

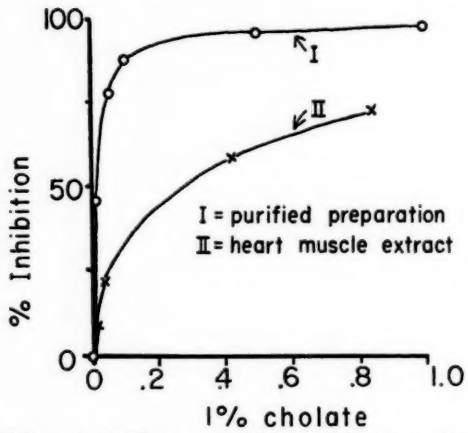


FIG. 2. Inhibition of cytochrome *c* oxidase activity by sodium cholate. The sodium cholate was added to the reaction mixture before the addition of the enzyme. The enzyme preparations were made and the cytochrome oxidase assays run as described under "Methods."

Only a very slight reduction of cytochromes *a* and a_3 results from the addition of ascorbate to a final concentration of 0.006 M in the absence of cytochrome *c* (Curve I of Fig. 3). In the steady state that results after the addition of cytochrome *c* (Curve II of Fig. 3), the peak at 605 mμ is 59 per cent ($D = 0.017/D = 0.029$) of the totally reduced value (Curve III); that at 445 mμ is only 24 per cent of the reduced value ($D = 0.047/D = 0.193$), and the peak at 550 mμ of cytochrome *c* is 40 per cent of the totally reduced figure ($D = 0.012/D = 0.031$). Curve IV of Fig. 3 shows that no further change in optical density results when $Na_2S_2O_4$ is added to the pigments that have been reduced when the mixture becomes anaerobic.

Higher concentrations of ascorbate (up to 0.02 M) will result in reduction of cytochromes *a* and a_3 to some extent (around 21 per cent reduction of peak at 605 mμ and 13 per cent reduction of peak at 445 mμ), even in the

absence of cytochrome *c*, but the oxygen uptake under these conditions is immeasurably small. The reduction of cytochromes *a* plus a_3 by ascorbate alone must be comparatively slow.

The addition of 0.004 M *p*-phenylenediamine to the preparation results in the reduction of cytochromes *a* and a_3 to a greater extent than with 0.02

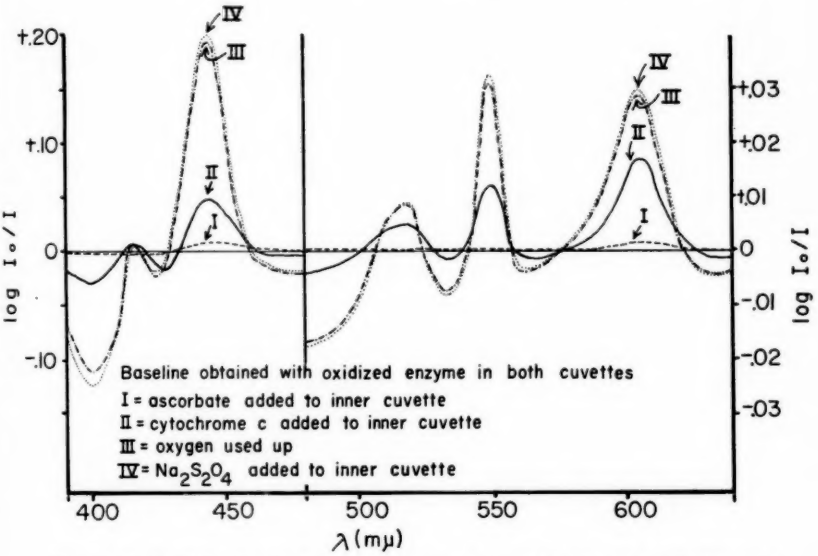


FIG. 3. Reduction of cytochromes $a + a_3$ by ascorbic acid and cytochrome *c*. The cytochrome *c* oxidase preparation was made as described under "Methods" and diluted 6-fold with 1 per cent cholate. The base-line was recorded with 3 ml. of oxidized enzyme in each cuvette, then neutralized ascorbic acid solution (0.05 ml. of 0.34 M) was added to the inner cuvette and the same volume of buffer was added to the reference cuvette and Curve I was recorded. Then cytochrome *c* (0.05 ml. of 140 μM) was added to give Curve II. After about 30 minutes, the mixture became anaerobic and Curve III was recorded. Curve IV was obtained after addition of solid $Na_2S_2O_4$.

M ascorbate, and the addition of cytochrome *c* then produced no further reduction of cytochromes *a* and a_3 in the steady state. The pigment formed on oxidation of *p*-phenylenediamine interferes to some extent with measurements of absorption spectra; hence it is more convenient to use a mixture of ascorbic acid and *p*-phenylenediamine. The ascorbic acid will then keep the *p*-phenylenediamine in the reduced form. Such an experiment is pictured in Fig. 4. Again the cytochromes *a* and a_3 are not reduced to the same extent in the steady state. The cytochrome *c* is usually

reduced to about the same extent as the peak at 605 mμ, but this varies somewhat and sometimes the cytochrome *c* is reduced to a smaller extent than the cytochrome *a*. This could be explained by the observation that the cytochromes *a* and a_3 can be reduced both directly by the reducing agents and by way of cytochrome *c*. It should be pointed out that the

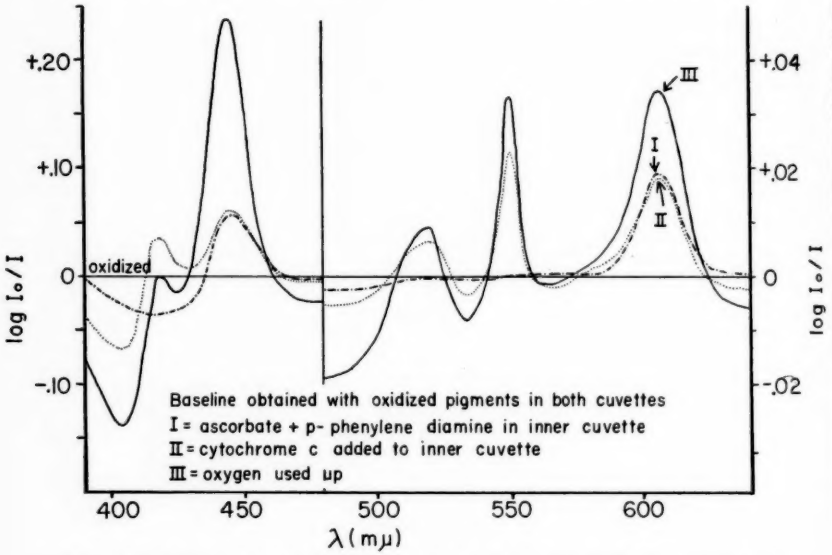


FIG. 4. Reduction of cytochromes $a + a_3$ by phenylenediamine + ascorbate + cytochrome *c*. Curves I, II, and III were obtained as described in the legend for Fig. 3, except that Curve II was obtained after addition of 0.03 ml. of a mixture of 0.34 M ascorbate plus 0.2 M *p*-phenylenediamine·2HCl, which had been neutralized to about pH 6. This made the final concentration in the reaction mixture 0.0034 M ascorbate and 0.002 M *p*-phenylenediamine. The final concentration of cytochrome *c* in the mixture was 2.3 μM and the time required for the oxygen concentration to reach zero was about 10 minutes.

changes in optical density at 605 and 445 mμ when the oxidized form of cytochrome *c* is reduced are so small that no correction need be made for the changes due to cytochrome *c* at the absorption peaks of cytochromes *a* and a_3.

Thus cytochromes *a* and a_3 can be reduced by ascorbic acid and *p*-phenylenediamine as well as by cytochrome *c*. The rate of direct reduction by ascorbic acid seems to be very slow, however. Also the rate of oxygen uptake in the presence of *p*-phenylenediamine is slower than that observed when both cytochrome *c* and *p*-phenylenediamine are added to the oxidase,

as shown by observations of the time required for the total reduction of the pigments as the oxygen is exhausted. This is difficult to explain, since the steady state levels of cytochromes *a* and a_3 are similar with and without cytochrome *c*. It could mean that the presence of cytochrome *c* reverses an inhibition of the reaction of the oxidase with oxygen. Traces A and B of Fig. 5 also show that the reduction of cytochromes *a* and a_3 to the steady state by *p*-phenylenediamine alone is slow compared to reduction in the presence of cytochrome *c* plus *p*-phenylenediamine.

Further experiments showed that cytochromes *a* and a_3 can also be reduced directly by hydroquinone, ferrocyanide, and cysteine, and with these reagents the extent of reduction in the steady state is similar to that observed with ascorbic acid. It should be emphasized that the reduction of the pigments by $Na_2S_2O_4$ is different from that observed with the other

TABLE II

Effect of Concentration of Sodium Cholate on Extent of Reduction of Bands at 605 and 445 mμ in Steady State

Sodium cholate concentration in reaction mixture	Reduction of band at 605 mμ	Reduction of band at 445 mμ
per cent	*per cent*	*per cent*
1.05	52	14
0.15	36	15
0.05	30	11
0.005		10–15

reagents described, since $Na_2S_2O_4$ removes the oxygen from the solution. With the other reagents, as long as oxygen is still present the pigments can apparently still be oxidized by oxygen. When cyanide was added to the preparation to bind the oxidase, the addition of any of the above reagents resulted in the production of the same spectrum that is observed when cyanide is added to the reduced pigments. The spectral changes resulting from the reaction of cytochromes *a* and a_3 with CO and cyanide will be described in a subsequent paper.

The cytochromes *a* and a_3 of the preparation described can also be reduced by the action of the heart muscle succinic oxidase system in the presence of added cytochrome *c*, provided that most of the ammonium sulfate is removed from the preparation by precipitating and resuspending it in 1 per cent cholate.

A few experiments were made with higher and lower concentrations of cholate. Table II shows the data from one series of experiments in which the cholate concentration was varied. The per cent reduction of the band at 605 mμ in the steady state in the presence of *p*-phenylenediamine and

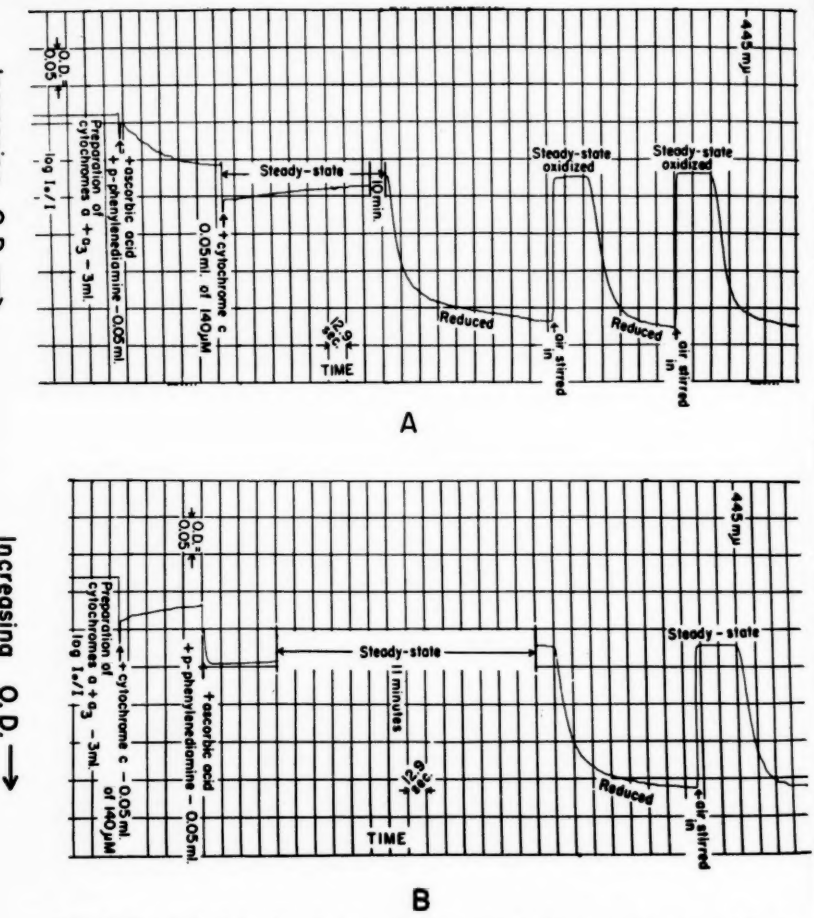


FIG. 5. The optical density changes at 445 mμ as cytochromes $a + a_3$ are oxidized and reduced. The purified cytochrome c oxidase preparation was diluted ten times with 1 per cent cholate. Changes in optical density at 445 mμ were traced on the chart of the recording spectrophotometer. The concentration of cytochrome c in the reaction mixture was 2.3 μM; the ascorbic acid and p-phenylenediamine were 0.0056 and 0.0011 M, respectively. In Trace A the ascorbate and p-phenylenediamine were added before the cytochrome c, while in Trace B the order of addition of these reagents was reversed.

cytochrome c decreased as the cholate concentration was lowered, but there was only a slight decrease in the band at 445 mμ. In the presence of stronger cholate (4 per cent or greater) it was observed that the cytochrome

c was nearly completely reduced in the steady state. At these cholate concentrations a shift in the position of the peaks of cytochromes *a* and a_3 to about 600 and 440 mμ was observed; thus no further experiments were done with cholate concentrations exceeding 1 per cent.

After reduction with cytochrome *c* plus *p*-phenylenediamine or by the heart muscle enzyme system, cytochromes *a* and a_3 are immediately reoxidized on stirring air into the mixture, then reduced again as the oxygen concentration falls to zero. This cycle of oxidation and reduction is illustrated in Fig. 5, where it can be seen that the oxidation of the pigments by oxygen is very rapid. This cycle can be easily recorded when the reactions are carried out in 1 per cent cholate, in which the turnover of the enzyme is decreased.

DISCUSSION

The data presented show that the combination of cytochromes *a* and a_3 can be directly reduced by ascorbic acid, *p*-phenylenediamine, hydroquinone, and ferrocyanide, as well as indirectly via cytochrome *c*. Although Borei and Björklund (11) concluded that a series of substituted phenylenediamines reacts with cytochrome *c* oxidase only through reaction with cytochrome *c*, their data do not seem to rule out the possibility that some direct reaction can take place. Under the conditions of our experiments, the rate of direct reduction of cytochromes *a* and a_3 is slow compared to that observed when cytochrome *c* is present. However, the rate with 0.01 M *p*-phenylenediamine is rapid enough so that a slow oxygen uptake is observed in the absence of cytochrome *c*. Keilin and Hartree (12) and Slater (13) observed that *p*-phenylenediamine could be oxidized by the heart muscle cytochrome oxidase system in the absence of added cytochrome *c*, while the other reducing agents tested were not oxidized. They suggest that the difference is due to rates of reduction of the endogenous cytochrome *c* by the different substances tested. The data presented here suggest that *p*-phenylenediamine could react directly with cytochromes *a* and a_3, but this does not agree with the observation of Keilin and Hartree that a heart muscle preparation which contained very little cytochrome *c* did not catalyze the oxidation of *p*-phenylenediamine unless cytochrome *c* was added. The possibility has not been excluded that the reducing agents tested can react with the purified pigments but not with those on the heart muscle particles.

A definite relationship was found between the height of the peaks of cytochromes *a* and a_3 of the oxidase preparation and the cytochrome *c* oxidase activity. The lack of correlation of activity and the peak at 601 mμ in the preparations of Eichel *et al.* (14) must have been due to the presence of varying amounts of deoxycholate in their reaction mixtures, as suggested by Wainio *et al.* (4).

The absorption spectra of cytochromes *a* and a_3 are different from those of other known hemochromogens. There is little or no apparent β-band, and the absorption of cytochrome *a* in the Soret region must be relatively small, or the oxidized and reduced peaks are unusually close together. This observation is somewhat reminiscent of cytochrome a_2, which shows very little increase in absorption in the Soret region of the spectrum when the oxidized form is reduced (15). Straub (16) pointed out that the relationship of the Soret peak of cytochrome a_3 to the visible peak would be more like that of other hemochromogens than would cytochrome *a*.

The peak in the absorption spectrum around 560 mμ ascribed by Ball *et al.* (17, 18) to cytochrome *c* oxidase is not present in our preparation, although the cytochrome *c* oxidase activity is comparable to that of the enzyme on the heart muscle particles.

The difference spectra observed during the aerobic steady state, which follows on addition of ascorbic acid and *p*-phenylenediamine to the preparation, leave no doubt now that the peaks at 605 and 444 mμ must represent more than one pigment, since the peaks are reduced to different extents in the steady state. However the observations are interpreted, the two peaks could not be the α- and γ-peaks of only one pigment. Paper II shows that cytochrome *a* can have an independent existence from cytochrome a_3.

Cholate in rather high concentrations (0.5 to 1 per cent) is strongly inhibitory to cytochrome *c* oxidase activity. This effect was also observed by Kiese and Reinwein (19) who did not make observations below a cholate concentration of 0.025 per cent. They concluded that the inhibition is proportional to the logarithm of the cholate concentration, but our data show that this relationship does not hold for lower concentrations of cholate. The inhibitory effect of cholate on the enzyme on the heart muscle particles is less than it is on the purified enzyme, and, what has not been recognized previously, the effect on the heart muscle enzyme is very different, depending upon whether the cholate is first added to the heart muscle extract, and the extract then diluted, or is added in the same final concentration to the reaction mixture. There may well be a number of substances in the heart muscle extract which will react with cholate.

The nature of the inhibition by cholate is not known. It may be that the reaction between cytochromes *a* and *c* is interfered with, since cytochromes *a* and a_3 are reduced to different extents in the steady state in the presence of 1 per cent cholate, and in cholate concentrations as high as 4 per cent the cytochrome *c* is nearly completely reduced in the steady state. The oxidation of cytochromes *a* and a_3 by oxygen is very rapid in the presence of 1 per cent cholate.

It should be stated that the experiments described here involve measurements of the aerobic steady state levels of reduced cytochromes *a*, a_3, and *c* and cannot be related to the experiments of Ball and Cooper (20), in

which the cytochromes on heart muscle particles were reduced in the absence of oxygen.

The experiments described do not show what parts of the two absorption peaks at 605 and 445 mμ correspond to cytochromes *a* and a_3, and the addition of any reagent which reacts with cytochrome a_3 during the steady state would result in a change of steady state. Further experiments will be necessary to establish this relationship.

SUMMARY

1. Two additional steps added to the previously described procedure for purification of cytochrome *c* oxidase yield a preparation, the spectrum of which shows only peaks at 605 and 445 mμ in the reduced form. The pigments present thus correspond to a mixture of cytochromes *a* plus a_3.

2. The cytochrome *c* oxidase activity of the preparation is proportional to the content of cytochromes *a* and a_3, and the relationship between the cytochrome *c* oxidase activity and the peak at 605 mμ is similar to the same ratio for the enzyme on heart muscle particles.

3. The ratio of the peaks at 445 and 605 mμ in a number of preparations was found to be fairly constant and gave an average value of 4.4.

4. The spectrum of cytochrome *a* appears to be different from that of most cytochromes; there is no β-band, and the increase in optical density on reduction in the Soret region is small in relation to that in the visible region of the spectrum.

5. The reduction of cytochromes *a* and a_3 was observed during the steady state that results after addition of cytochrome *c* and *p*-phenylenediamine while the oxygen concentration slowly falls to zero; then the total reduction of the cytochromes was observed. It was found that the peak at 605 mμ was nearer the reduced value in the steady state than that at 445 mμ. Thus the two peaks could not be those of only one pigment.

6. Other steady state data showed that the cytochromes *a* and a_3 of the preparation can be reduced directly by ascorbic acid, *p*-phenylenediamine, cysteine, ferrocyanide, or reduced cytochrome *c*. The reduction by cytochrome *c* is a much more rapid reaction.

7. The inhibition of cytochrome *c* oxidase activity by sodium cholate is described.

8. Even in the presence of sodium cholate, which inhibited the oxidation of cytochrome *c* by the preparation, the cytochromes *a* and a_3 are seen to be rapidly oxidized by oxygen.

BIBLIOGRAPHY

1. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127**, 167 (1939).
2. Chance, B., *J. Biol. Chem.*, **202**, 397 (1953).
3. Chance, B., *Nature*, **169**, 215 (1952).

4. Wainio, W. W., Person, P., and Eichel, B., *J. Biol. Chem.*, **192,** 349 (1951).
5. Lundegardth, H., *Nature*, **171,** 521 (1953).
6. Smith, L., and Stotz, E., *J. Biol. Chem.*, **209,** 819 (1954).
7. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124,** 397 (1938).
8. Chance, B., *J. Biol. Chem.*, **197,** 557 (1952).
9. Yang, C. C., and Legallais, V., *Rev. Scient. Instruments*, **25,** 801 (1954).
10. Smith, L., *Arch. Biochem. and Biophys.*, **50,** 285 (1954).
11. Borei, H. G., and Björklund, U., *Biochem. J.*, **54,** 357 (1953).
12. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **125,** 171 (1938).
13. Slater, E. C., *Biochem. J.*, **44,** 305 (1949).
14. Eichel, B., Wainio, W. W., Person, P., and Cooperstein, S. J., *J. Biol. Chem.*, **183,** 89 (1950).
15. Smith, L., *Arch. Biochem. and Biophys.*, **50,** 299 (1954).
16. Straub, F. B., *Z. physiol. Chem.*, **268,** 227 (1941).
17. Ball, E. G., Strittmatter, C. F., and Cooper, O., *J. Biol. Chem.*, **193,** 635 (1951).
18. Ball, E. G., and Cooper, O., *J. Biol. Chem.*, **198,** 629 (1952).
19. Kiese, M., and Reinwein, D., *Biochem. Z.*, **324,** 51 (1953).
20. Ball, E. G., and Cooper, O., *Biol. Bull.*, **99,** 317 (1950).

REACTIONS OF CYTOCHROMES *a* AND a_3*

II. STUDIES WITH MICROCOCCUS PYOGENES VAR. ALBUS AND BACILLUS SUBTILIS

By LUCILE SMITH

(From the Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, August 16, 1954)

Since it has not so far been possible to separate heart muscle cytochromes *a* and a_3 and thus to study the properties of the two pigments, another approach to this problem was found in a study of cells containing a different cytochrome make-up from that found in heart muscle. It was found that *Micrococcus pyogenes* var. *albus* seemed to contain cytochrome *a*, but no cytochrome a_3 (1–3); thus in these cells the reactions of cytochrome *a* could be studied in the absence of cytochrome a_3. In *Bacillus subtilis* there was observed a different ratio of cytochrome *a* to cytochrome a_3 from that present in heart muscle extracts (1, 4). This paper describes the absorption spectra of these two organisms and the changes in absorption spectra which result from reaction with carbon monoxide and cyanide.

Methods

Preparation of Bacterial Suspensions—M. pyogenes var. *albus* was obtained from Dr. D. J. O'Kane, to whom our thanks are due, and the culture of *B. subtilis* was kindly supplied by Professor D. Keilin. The bacteria were grown at 35° for 24 hours on a medium of the following composition: 10 gm. of NaCl, 8 gm. of Difco nutrient broth, and 20 gm. of Bacto-agar per liter of distilled water. The bacteria were washed from the plates with a solution containing 1.5 gm. of Na_2HPO_4, 0.75 gm. of KH_2PO_4, 0.2 gm. of $MgSO_4 \cdot 7H_2O$, and 0.05 gm. of NaCl per liter of distilled water. They were then washed four times with the same solution. In a few experiments *M. pyogenes* var. *albus* was grown on a medium composed of 10 gm. of Difco yeast extract, 5 gm. of glucose, 5 ml. of glycerol, 5 ml. of sodium lactate (60 per cent), 2 gm. of $(NH_4)_2SO_4$, 2 gm. of KH_2PO_4, 0.5 gm. of Na_2HPO_4, 2 gm. of $MgCl_2$, 0.02 gm. of $MnSO_4$, 0.2 gm. of tryptophan, 0.15 gm. of cystine, 2 mg. of *p*-aminobenzoic acid, 2 mg. of nicotinic acid, 2 mg. of calcium pantothenate, and 20 gm. of agar per liter of distilled water. The pH of the medium was adjusted to 6.5. In the experi-

* This research was supported by a grant from the Division of Grants and Fellowships, National Institutes of Health, United States Public Health Service.

ments to be described this medium is referred to as the special yeast extract medium.

Broken cell suspensions of the bacteria were prepared by exposing the cells to sonic vibrations in a 9000 cycle Raytheon instrument. The *B. subtilis* was exposed for 15 minutes and the *M. pyogenes* for 90 minutes, after which any whole cells remaining were removed by centrifugation at 5000 r.p.m. for 10 minutes in an angle centrifuge. For several experiments this supernatant fluid was centrifuged for 30 minutes at 140,000 × *g*, and the pellet of insoluble material obtained was washed once with the weak buffer solution described above, then suspended in the same solution. Suspension of the insoluble pellet was accomplished by means of a hand-driven ground glass homogenizer. This suspension is referred to as the "washed insoluble fraction."

Measurement of Difference Spectra—The difference between the spectrum of the bacterial pigments in the reduced form and in the oxidized or steady state oxidized form was recorded by means of a specially designed spectrophotometer (2, 5), which automatically records the difference between the spectra of two forms of pigments in two different cuvettes. The pigments were reduced by adding substrate (glucose for *M. pyogenes* and lactate for *B. subtilis*) and allowing the cells to respire and become anaerobic. To keep the cells in the oxidized form, no substrate was added, and oxygen was bubbled through the cell suspension. The difference spectrum of the carbon monoxide compound or of the cyanide or methyl hydrogen peroxide compounds was obtained similarly after either bubbling carbon monoxide through the suspension for 30 seconds or adding strong KCN (10 μl. of 1.5 M to 3 ml. of suspension) or MeOOH (10 μl. of 20 mM[1] solution to 3 ml. of the cell suspension).

Measurement of Catalase Activity—Catalase activity was determined on broken cell suspensions from which any unbroken cells had been removed by centrifugation. The method of Bonnichsen, Chance, and Theorell (6) was used for the assays. The procedure was found to be unsatisfactory with suspensions of whole cells, since the velocity constant for the decomposition of H_2O_2 increased with time.

Manometric Experiments—The inhibition of respiration by cyanide was measured manometrically with the usual Warburg technique (7).

Results

Difference Spectra of M. pyogenes Var. albus

Reduced Minus Oxidized Difference Spectrum—Fig. 1, Curve A, shows the difference spectrum of the pigments of *M. pyogenes* in the reduced

[1] mM represents millimolar (millimoles per liter) and μM micromolar (micromoles per liter) concentration.

form compared with the steady state oxidized form of the pigments as a base-line. It can be seen that there are peaks in the absorption spectrum in the visible region at the same wave-lengths as those of the cytochromes of yeast or heart muscle: 605, 565, 552, and 523 mμ. However, there is no evidence in the Soret region of a peak corresponding to cytochrome a_3 at 445 mμ (2).

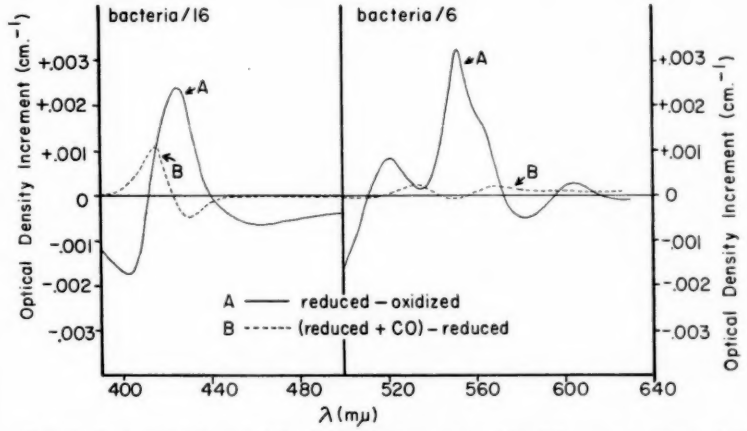


FIG. 1. Reduced minus oxidized and carbon monoxide difference spectra of *M. pyogenes* var. *albus*. To obtain the reduced-oxidized difference spectrum (Curve A), the base-line was obtained with oxidized cells (no substrate added and cells bubbled with oxygen) in both cuvettes. Then glucose was added to one cuvette, the cells were allowed to become anaerobic, and the spectrum was plotted. To obtain Curve B, the base-line was recorded with reduced pigments (anaerobic cells) in both cuvettes; then CO was bubbled through one cuvette for 30 seconds and the difference spectrum was recorded. The cell suspension was diluted six times for measurements in the visible region of the spectrum and sixteen times for experiments in the Soret region.

Carbon Monoxide Difference Spectrum—When carbon monoxide is added to the reduced pigments of *M. pyogenes* (anaerobic cells) and the resulting spectrum is compared with that of the reduced pigments in the reference cuvette, the difference spectrum shown in Fig. 1, Curve B, is obtained. Peaks in the absorption spectrum appear at about 568, 533, and 415 mμ, with troughs at about 557 and 430 mμ. No change in the spectrum is observed in the region of the peak of cytochrome *a*, and the peaks formed are at quite different wave-lengths from those of the cytochrome a_3-carbon monoxide compound, which are at about 590, 540, and 430 mμ (8). The cytochrome in *M. pyogenes* which reacts with carbon monoxide has been shown to be a new type of respiratory enzyme (9).

Reaction with Cyanide—When cyanide is added to a suspension of anaerobic *M. pyogenes*, in which the cytochromes are in the reduced form, and the spectrum is compared with the oxidized cells as a blank (Fig. 2, Curve B), there appears to be a shift in the peak of cytochrome *a* from 605 mμ to about 595 mμ. However, examination of the cyanide difference spectrum obtained when the cytochromes are reduced in the reference cuvette (Fig. 2, Curve C) shows that the trough in the difference spectrum in the

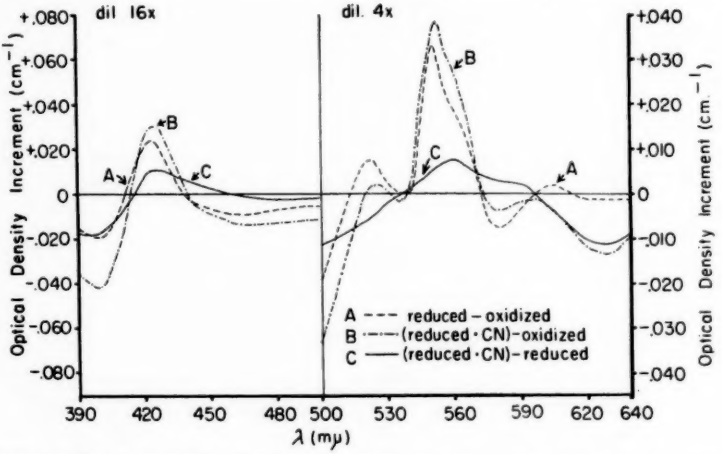


FIG. 2. Cyanide difference spectra of *M. pyogenes* var. *albus*. Curve A was obtained as described for Fig. 1. The bacterial suspension was diluted four times for experiments in the visible region of the spectrum and sixteen times for experiments in the Soret region. To obtain Curve B, KCN was added to anaerobic cells in one cuvette (final concentration = 5×10^{-3} M) and the difference spectrum was plotted with oxidized cells in the reference cuvette. Curve C was obtained similarly with reduced pigments (anaerobic cells) in the reference cuvette.

region of the peak of cytochrome *a* is at about 630 mμ, rather than at 605 mμ, and thus some pigment other than cytochrome *a* reacted with the cyanide. The peaks in the difference spectrum shown in Curve C are at about 590, 557, and 428 mμ, and there is a large trough around 400 mμ in the Soret region of the spectrum. The peaks and troughs are those which would be expected if the spectral changes were due to the reaction of cyanide with catalase; peaks in the absorption spectrum of the bacterial catalase-cyanide compound are reported to be at 592, 555.5, and 426 mμ (10, 11). Other evidence agrees with the assumption that the changes observed on addition of cyanide are due to its reaction with the bacterial catalase.

The difference spectrum obtained when methyl hydrogen peroxide is added to anaerobic cells of *M. pyogenes*, shown in Fig. 3, Curve B, is similar to the difference spectrum of the catalase-methyl hydrogen peroxide compound I, as calculated from the data of Chance and Herbert (11), pictured in Fig. 4. It was shown by Chance (12) that in anaerobic cells of *Micrococcus lysodeikticus* the catalase is in the form of the uncombined

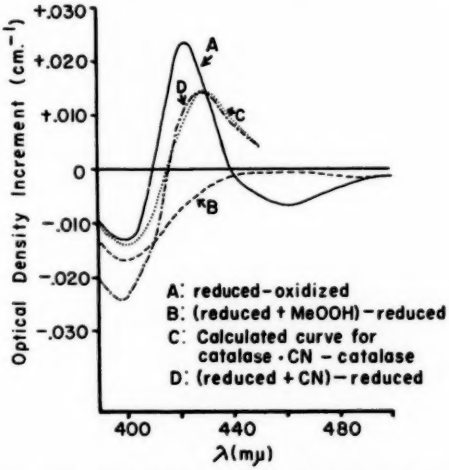


FIG. 3. Difference spectra in Soret region after addition of CH_3OOH or KCN to *M. pyogenes* var. *albus*. Curve A represents the reduced-oxidized difference spectrum as in Figs. 1 and 2. The difference spectrum obtained when CH_3OOH (final concentration = 67 μM) was added to the anaerobic cells and the spectrum was compared with anaerobic cells in the reference cuvette is shown in Curve B. From the data of Chance and Herbert (11) the difference spectrum corresponding to a similar concentration of the catalase-cyanide compound was calculated; this is depicted in Curve C. Curve D represents the difference spectrum obtained by adding cyanide to anaerobic cells and comparing them with anaerobic cells in the reference cuvette.

pigment. Curve C of Fig. 3 is the calculated cyanide difference spectrum that would correspond to the observed amount of catalase-CH_3OOH compound. The calculated peak is almost identical with that obtained on addition of cyanide to another sample of the same cell suspension[2] (Curve D of Fig. 3).

[2] The trough around 400 mμ in the cyanide difference spectrum is often observed to be larger than that calculated to correspond to the catalase present, as in the experiment illustrated. The observation that this trough and the one around 450 mμ vary considerably with the oxygen tension seems to indicate that the variability in the region of these troughs might be due to the flavoprotein present.

The catalase concentration can be calculated from the CH_3OOH difference spectrum, by using the value $\Delta\epsilon = -200$ cm.$^{-1}$ × mM^{-1} at 400 mμ, as calculated from the data of Chance and Herbert (11). The concentrations calculated this way with broken cell suspensions were checked by the titration method, with fairly good agreement. The following are typical data: (*a*) catalase calculated from difference spectrum = 0.135 μM; (*b*) catalase determined by titration method on same suspension, concentration = 0.12 μM.

The addition of cyanide to cells in which the catalase present was bound in the form of the CH_3OOH compound produced no change in spectrum.

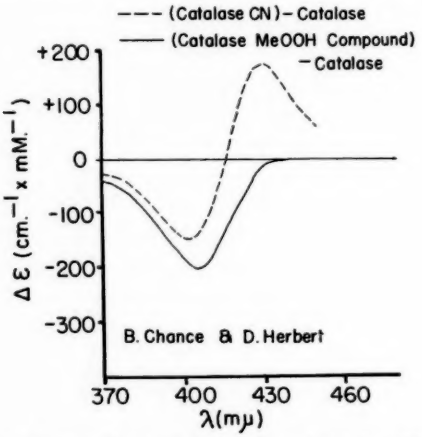


FIG. 4. Difference spectra of catalase-cyanide and catalase-MeOOH compounds. The difference spectra are calculated from the data of Chance and Herbert (11).

When the *M. pyogenes* was grown on the special yeast extract medium described under "Methods," the washed cells appeared to contain no catalase, since no change in spectrum was observed on addition of MeOOH to the anaerobic cells. These cells showed also no change in spectrum upon addition of cyanide.

It was not possible to starve the cells of *M. pyogenes* so that there was no endogenous substrate left; even after repeated washing and aeration, the cytochromes became reduced immediately upon addition of cyanide, which must inhibit their oxidation. It is thus not possible to investigate the spectral changes resulting from a reaction of cyanide with the oxidized form of the pigments in the whole cells. It was observed with intact cells that the same difference spectrum was obtained on addition of cyanide to the pigments reduced by the action of the cellular dehydrogenases as that obtained after reduction by addition of $Na_2S_2O_4$. Spectral changes like

those observed by Keilin and Hartree (8) when cyanide was added to heart muscle extract in which the cytochromes were reduced in the absence of air are not seen in *M. pyogenes*. Catalase cannot be reduced by $Na_2S_2O_4$.

A few experiments were made with the "washed insoluble fraction" of *M. pyogenes*, which contains no catalase, but shows typical reduced-oxidized and carbon monoxide difference spectra. Addition of cyanide to this suspension with the pigments in the oxidized form resulted in only a small generalized decrease in the optical density between about 450 to 390 mμ, with no appearance of peaks or troughs. From all of the observations described, there is no evidence that any pigment in *M. pyogenes* except catalase reacts with cyanide to produce a pronounced change in absorption spectrum.

Some component in the system of enzymes involved in the respiration of these bacteria must react with cyanide, since the manometric experiments showed the respiration to be cyanide-sensitive: 78 per cent inhibition of respiration was observed in the presence of 10^{-4} M cyanide. The addition of cyanide to the cells prevents the formation of the carbon monoxide compound; however, the formation of the catalase-CH_3OOH compound does not inhibit the reaction with carbon monoxide. This seems to indicate that catalase is not the cyanide-combining compound involved in respiration.

Difference Spectra of B. subtilis

Reduced Minus Oxidized Difference Spectrum—Curve A of Fig. 5 shows the difference spectrum of the *B. subtilis* pigments with the steady state oxidized form of the pigments as a reference. The peaks in the difference spectrum are at the same wave-lengths as those seen in the difference spectrum of yeast or heart muscle (13): at 605, 564, 552, 523, 445, and 425 mμ. However, in *B. subtilis* the ratio of the increase in optical density on reduction at 445 mμ to that at 605 to 620 mμ is 2, whereas the ratio in yeast is 4 (1).

Reaction with Carbon Monoxide—Chance (2) has described the carbon monoxide difference spectrum (carbon monoxide compound minus the reduced pigment) of *B. subtilis* in the Soret region of the spectrum. Curve B of Fig. 5 shows such a difference spectrum in both the Soret and visible regions. The troughs and peaks in the difference spectrum indicate that a reduced pigment with peaks around 605 and 445 mμ (wave-lengths of troughs in the difference spectrum) reacted to form a compound with peaks at 590, 540, and 430 mμ. These wave-lengths are characteristic of the spectrum of a carbon monoxide compound of a cytochrome of type a_3. However, the data of Chance (4) suggest that the cytochrome a_3 of *B. subtilis* is different from that of yeast and muscle, since the ratios of the α- to the γ-bands of the carbon monoxide compound are quite different. From observations of the optical density changes in the presence of CO,

he calculated that the molar ratio of cytochrome a_3 to cytochrome *a* in *B. subtilis* is 2.3, as compared with the values of 0.7 and 1.1 for heart muscle and yeast, respectively. Since the ratio of the optical density change on reduction at 445 mμ to that at 605 − 620 mμ is 2 for *B. subtilis* and 4 for yeast, this would seem to indicate that the ratio of the γ- to α-bands of cytochrome a_3 in *B. subtilis* is different from that in yeast, as it is for the CO compounds.

Reaction with Methyl Hydrogen Peroxide—The difference spectrum obtained when CH_3OOH is added to anaerobic cells of *B. subtilis*, like that

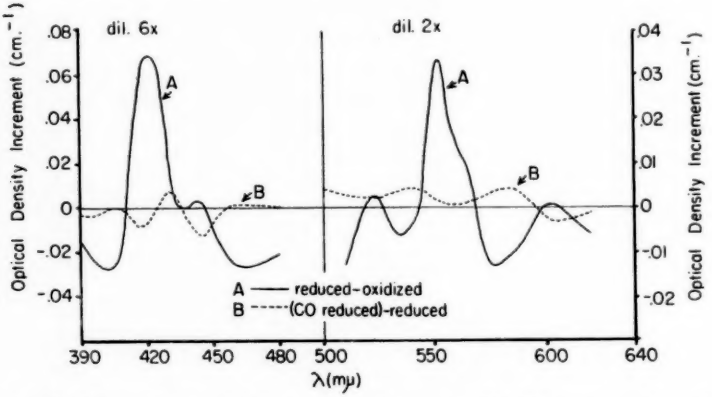


FIG. 5. Reduced minus oxidized and carbon monoxide difference spectra of *B. subtilis*. Curve A was obtained with oxidized cells (no substrate added and oxygen passed through the suspension) in the reference cuvette and anaerobic cells in the other cuvette. The cell suspension was diluted two times for measurements in the visible region of the spectrum and six times for experiments in the Soret region. To obtain Curve B, the reference cuvette contained anaerobic cells and the other cuvette contained anaerobic cells through which pure CO was passed for 30 seconds.

observed with *M. pyogenes*, is typical of the catalase-CH_3OOH compound I. The concentration of catalase in the intact cells can be calculated from this difference spectrum, as described above. When the cells are then ruptured by exposure to sonic vibration and the catalase content of the suspension is measured by the titration method, the two values agree rather closely. Typical data are as follows: (*a*) spectrophotometric method, catalase concentration in intact cells = 0.81 μM; (*b*) titration of broken cell suspension, catalase concentration = 0.73 μM. Nearly 100 per cent of the cells of *B. subtilis* are broken in the treatment with sonic vibration. Thus the measurement of the CH_3OOH difference spectrum of whole cells gives a valid measurement of the catalase content.

Reaction with Cyanide—The spectral changes resulting from the reaction of the pigments of *B. subtilis* with cyanide are depicted in Fig. 6. The difference spectrum obtained when cyanide is added to the anaerobic cells and compared with anaerobic cells in the reference cuvette (Curve C) is similar to that obtained with *M. pyogenes*, except that there is also a small trough at 445 mμ in the *B. subtilis* spectrum and the peak at 590 mμ is more

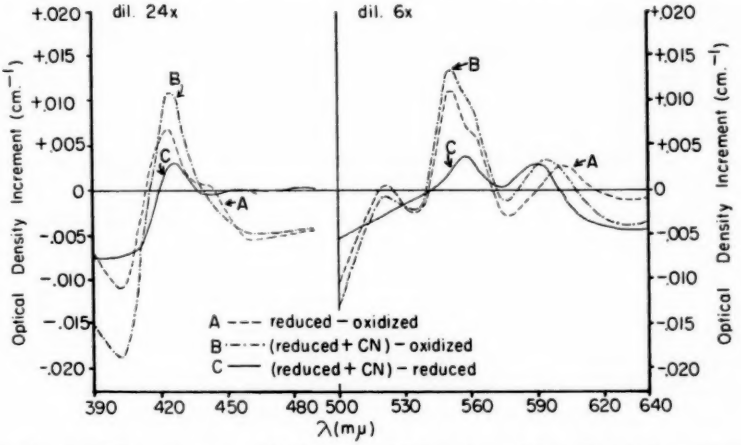


FIG. 6. Difference spectra obtained on addition of cyanide to *B. subtilis*. Curve A represents the reduced-oxidized difference spectrum obtained with oxidized pigments (oxygenated cells with no substrate added) in the reference cuvette and anaerobic cells in the other cuvette. The cell suspension was diluted six times for measurements in the visible region of the spectrum and twenty-four times for those in the Soret region. Curve B was obtained when KCN (final concentration = 5×10^{-3} M) was added to the anaerobic cells and the reference cuvette contained oxidized cells. In the experiment depicted in Curve C the spectrum of the reduced cells plus cyanide was compared with that of the reduced (anaerobic) cells in the reference cuvette.

pronounced. In addition to the reaction of catalase with cyanide in *B. subtilis* there is a change in spectrum due to the cytochrome a_3 present. This change at 445 mμ is similar to that observed when cyanide is added to a suspension of heart muscle particles (8), where the peak of cytochrome a_3 at 445 mμ decreases on addition of cyanide.

DISCUSSION

The observations described on the difference spectra of the pigments of *M. pyogenes* var. *albus* and *B. subtilis* show several new facets of the relationship between cytochrome *a* and cytochrome a_3.

Cytochromes *a* and a_3 do not invariably occur together; thus the mere presence of an absorption band at 605 mμ in the reduced form in cells or extracts cannot be taken as evidence for the presence of cytochrome a_3.

The separate existence of an absorption band at 605 mμ without one at 445 mμ is in disagreement with the assumption made by some (14) that the absorption bands at 605 and 445 mμ are in reality the α- and γ-bands of only one compound and that the band at 605 mμ is the α-band of cytochrome *c* oxidase.

As shown by Keilin and Hartree (8), cytochrome *a* does not react with either carbon monoxide or cyanide. The peak of cytochrome *a* at 605 mμ could not then be that of the oxidase, since the oxidase reacts with carbon monoxide. It cannot be assumed that the bacterial cytochrome *a* is identical with yeast or heart muscle cytochrome *a*, particularly since the cytochrome a_3 of *B. subtilis* has been shown to have some different properties from those of the cytochrome a_3 of yeast or heart muscle. The reactions of *B. subtilis* cytochrome a_3 with carbon monoxide and cyanide, however, give the same qualitative spectral changes as yeast or heart muscle cytochrome a_3.

The ratio of cytochromes *a* to a_3 in *B. subtilis* is different from that in yeast or heart muscle. As suggested by Chance (4), this makes less plausible the attractive hypothesis of Ball and Cooper (15) that oxygen reacts with a complex composed of 3 molecules of cytochrome *a* and 1 of cytochrome a_3. This hypothesis was based on the assumption that cytochromes *a* and a_3 have identical extinction coefficients at 605 mμ and that the pigments exist in a 3:1 ratio. The data reported in the preceding paper (16) make this assumption seem uncertain.

The carbon monoxide difference spectrum of *M. pyogenes* var. *albus* throughout the visible and Soret regions of the spectrum agrees with the carbon monoxide action spectrum (9). Apparently this new type of respiratory enzyme is the only pigment in these bacteria which reacts with carbon monoxide.

The experiments reported in this paper give no information concerning the changes in absorption spectrum that accompany the reaction of cytochrome a_3 with cyanide, except to confirm the observation that the peak of reduced cytochrome a_3 at 445 mμ disappears on addition of cyanide. Further observations on the reaction of cytochrome a_3 with cyanide will be reported in another paper.

The changes in absorption spectrum that result from the addition of carbon monoxide or cyanide to the bacterial cells emphasize the importance of the formation of the proper carbon monoxide compound to establish the identity of a cytochrome a_3 or of any other oxidase. Any catalase or peroxidase present will react with cyanide, but not with carbon monoxide, since these enzymes are not reduced physiologically.

Calculations of the catalase content of *B. subtilis* from the spectral changes observed on addition of cyanide or of CH_3OOH yield essentially the same value as that obtained by titration of the broken cell suspensions. This gives added evidence that measurements of difference spectra of various kinds in intact cells give valid measurements of the pigments present.

SUMMARY

Studies were made of the spectrophotometric changes following addition of carbon monoxide or cyanide to *Micrococcus pyogenes* var. *albus*, which contains cytochrome *a*, but no cytochrome a_3, and to *Bacillus subtilis*, which contains a different ratio of cytochrome *a* to cytochrome a_3 from that in heart muscle. The data indicate the following.

1. Cytochromes *a* and a_3 do not invariably occur together.

2. No change in the absorption spectrum of cytochrome *a* results from the addition of carbon monoxide or cyanide.

3. The only compound in *M. pyogenes* var. *albus* which reacts with carbon monoxide is a new type of respiratory enzyme.

4. Although there appears to be a shift in the peak of cytochrome *a* at 605 mμ on addition of cyanide to *M. pyogenes* var. *albus*, the changes in the absorption spectrum which result from the reaction with cyanide are those due to the reaction with the catalase present. In *B. subtilis*, in addition, a disappearance of the peak of cytochrome a_3 at 445 mμ results from the addition of cyanide.

5. The concentration of catalase in a suspension of intact bacteria can be determined by measuring the formation of the catalase-CH_3OOH compound I with a sensitive spectrophotometric instrument.

BIBLIOGRAPHY

1. Smith, L., *Arch. Biochem. and Biophys.*, **50**, 299 (1954).
2. Chance, B., *J. Biol. Chem.*, **202**, 383 (1953).
3. Chance, B., *J. Biol. Chem.*, **202**, 397 (1953).
4. Chance, B., *J. Biol. Chem.*, **202**, 407 (1953).
5. Yang, C. C., and Legallais, V., *Rev. Scient. Instruments*, **25**, 801 (1954).
6. Bonnichsen, R. D., Chance, B., and Theorell, H., *Acta chem. Scand.*, **1**, 685 (1947).
7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1947).
8. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127**, 167 (1939).
9. Chance, B., Smith, L., and Castor, L. N., *Biochim. et biophys. acta*, **12**, 289 (1953).
10. Herbert, D., and Pinssent, J., *Biochem. J.*, **43**, 193 (1948).
11. Chance, B., and Herbert, D., *Biochem. J.*, **46**, 402 (1950).
12. Chance, B., *Science*, **116**, 202 (1952).
13. Chance, B., *Nature*, **169**, 215 (1952).
14. Person, P., Wainio, W. W., and Eichel, B., *J. Biol. Chem.*, **202**, 369 (1953).
15. Ball, E. G., and Cooper, O., *J. Biol. Chem.*, **198**, 629 (1952).
16. Smith, L., *J. Biol. Chem.*, **215**, 833 (1955).

XUM

INDEX TO AUTHORS

A

Abendschein, Patricia A. See *Meister, Levintow, Greenfield, and Abendschein*, 441

Abrams, Adolph. See *Borsook, Abrams, and Lowy*, 111

Abramsky, Tessa. See *Shemin, Russell, and Abramsky*, 613

Aisenberg, A. C., and **Potter, Van R.** Effect of fluoride and dinitrophenol on acetate activation in kidney and liver homogenates, 737

Ashmore, James, Renold, Albert E., Nesbett, Frances B., and **Hastings, A. Baird.** Studies on carbohydrate metabolism in rat liver slices. V. Glycerol metabolism in relation to other substrates in normal and diabetic tissue, 153

B

Baxter, Robert F. See *Mounter, Baxter, and Chanutin*, 699

Beatty, Clarissa H., and **West, Edward S.** Effect of succinic and malic acids and fructose on ketosis in alloxan-diabetic rats, 661

Bernstein, I. A., Lentz, Kenneth, Malm, Mignon, Schambye, Per, and **Wood, Harland G.** Degradation of glucose-C^{14} with *Leuconostoc mesenteroides;* alternate pathways and tracer patterns, 137

Bishop, Jillian. See *Buston and Bishop*, 217

Bloom, Ben. Fraction of glucose catabolized via the Embden-Meyerhof pathway: alloxan-diabetic and fasted rats, 467

—, **Eisenberg, Frank, Jr.,** and **Stetten, DeWitt, Jr.** Glucose catabolism in liver slices via the phosphogluconate oxidation pathway, 461

Borgström, Bengt, Sudduth, Herschel C., and **Lehninger, Albert L.** Phosphorylation coupled to reduction of cytochrome *c* by β-hydroxybutyrate, 571

Borsook, Henry, Abrams, Adolph, and **Lowy, Peter H.** Fructose-amino acids in liver: stimuli of amino acid incorporation *in vitro*, 111

Bott, P. A. The concentration of potassium in glomerular urine of *Necturi*, 287

Boyer, P. D. See *Harrison, Boyer, and Falcone*, 303

Braun, Armin C. See *Woolley, Schaffner, and Braun*, 485

Brown, Douglas M. See *Smith, Kimmel, Brown, and Thompson*, 67

Bueding, Ernest, and **MacKinnon, Joan A.** Hexokinases of *Schistosoma mansoni*, 495

— and —. Studies of the phosphoglucose isomerase of *Schistosoma mansoni*, 507

Buston, H. W., and **Bishop, Jillian.** Synthetic α-amino-β-hydroxycaproic acids, 217

C

Campbell, Mildred. See *Hess, Campbell, and Herranen*, 163

Chaikoff, I. L. See *Tong and Chaikoff*, 473

Chanutin, Alfred. See *Mounter, Baxter, and Chanutin*, 699

—. See *Mounter, Dien, and Chanutin*, 691

Chargaff, Erwin. See *Spitnik, Lipshitz, and Chargaff*, 765

Cheldelin, Vernon H. See *Stewart, Cheldelin, and King*, 319

Clark, Carroll T. See *Udenfriend, Weissbach, and Clark*, 337

Cook, Margaret. See *Lorber and Cook*, 823

Coon, Minor J. See *Rose, Coon, Lockhart, and Lambert*, 101

Cotzias, George C. See *Serlin and Cotzias*, 263

XUM

Crampton, Charles F., Moore, Stanford, and **Stein, William H.** Chromatographic fractionation of calf thymus histone, 787

D

D'Angeli, F. See *Henderson, Koski, and D'Angeli,* 369

Dannenberg, Arthur M., Jr., and **Smith, Emil L.** Action of proteinase I of bovine lung. Hydrolysis of the oxidized B chain of insulin; polymer formation from amino acid esters, 55

— and —. Proteolytic enzymes of lung, 45

Deiss, William P., and **Leon, Arthur S.** Mucopolysaccharides of heart valve mucoprotein, 685

Dien, Lien Tien H. See *Mounter, Dien, and Chanutin,* 691

Dorfman, Ralph I. See *Forchielli, Rosenkrantz, and Dorfman,* 713

E

Eisenberg, Frank, Jr. See *Bloom, Eisenberg, and Stetten,* 461

Eisenstein, Albert B. Metabolism of cortisone by rat liver homogenate, 585

F

Falcone, A. B. See *Harrison, Boyer, and Falcone,* 303

Fellig, Josef. See *Thannhauser, Fellig, and Schmidt,* 211

Finch, Clement A. See *Gabrio, Hennessey, Thomasson, and Finch,* 357

Fishman, William H., and **Green, Sidney.** Microanalysis of glucuronide glucuronic acid as applied to β-glucuronidase and glucuronic acid studies, 527

Forchielli, Enrico, Rosenkrantz, Harris, and **Dorfman, Ralph I.** Metabolism of 11-deoxycortisol *in vitro,* 713

Fresco, Jacques R., and **Warner, Robert C.** Application of the isotope derivative method to the analysis of pyrimidines, 751

Fritzson, Per. See *Pihl and Fritzson,* 345

Futterman, Sidney, and **Roe, Joseph H.** The identification of ribulose and L-xylulose in human and rat urine, 257

G

Gabrio, Beverly Wescott, Hennessey, Marion, Thomasson, Joan, and **Finch, Clement A.** Erythrocyte preservation. IV. *In vitro* reversibility of the storage lesion, 357

Gray, Irving. See *Jordan and Gray,* 669

Green, Sidney. See *Fishman and Green,* 527

Greenberg, David M. See *Matsuo and Greenberg,* 547

Greenfield, Robert E. See *Meister, Levintow, Greenfield, and Abendschein,* 441

Guarino, Armand J., and **Sable, Henry Z.** Studies on phosphomutases. II. Phosphoribomutase and phosphoglucomutase, 515

H

Harrison, W. H., Boyer, P. D., and **Falcone, A. B.** The mechanism of enzymic phosphate transfer reactions, 303

Hastings, A. Baird. See *Ashmore, Renold, Nesbett, and Hastings,* 153

Hayaishi, Osamu. Enzymatic decarboxylation of malonic acid, 125

Henderson, L. M., Koski, Ruth E., and **D'Angeli, F.** The rôle of riboflavin and vitamin B_6 in tryptophan metabolism, 369

Hennessey, Marion. See *Gabrio, Hennessey, Thomasson, and Finch,* 357

Herranen, Ailene. See *Hess, Campbell, and Herranen,* 163

Hess, E. L., Campbell, Mildred, and **Herranen, Ailene.** A protease present in extracts of a lymphatic organ, 163

XUM

Huffman, Max N., and **Lott, Mary Harriet.** 16-Substituted steroids. XII. Estradiol-3,16α, 627
— and —. XIII. Androstan-3β,16β-diol and androstan-3β,16α-diol, 633
Hutcheson, Ruth M. See *Touster, Hutcheson, and Rice,* 677

J

Johnson, B. Connor. See *Sime and Johnson,* 41
Jordan, William J., and **Gray, Irving.** The biochemical response to trauma. I. Nucleotide changes in tourniquet shock, 669

K

Kaplan, Nathan O. See *Shuster and Kaplan,* 183
—. See *Shuster, Kaplan, and Stolzenbach,* 195
Kimmel, J. R. See *Smith, Kimmel, Brown, and Thompson,* 67
King, Tsoo E. See *Stewart, Cheldelin, and King,* 319
Korn, Edward D. Clearing factor, a heparin-activated lipoprotein lipase. I. Isolation and characterization of the enzyme from normal rat heart, 1
—. II. Substrate specificity and activation of coconut oil, 15
Kornberg, Arthur, Lieberman, Irving, and **Simms, Ernest S.** Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate, 389
—, —, and —. Enzymatic synthesis of purine nucleotides, 417
—. See *Lieberman, Kornberg, and Simms,* 403, 429
Koski, Ruth E. See *Henderson, Koski, and D'Angeli,* 369
Kratzer, F. Howard. See *Rachele, Kuchinskas, Kratzer, and du Vigneaud,* 593
Kuchinskas, Edward J. See *Rachele, Kuchinskas, Kratzer, and du Vigneaud,* 593

L

Lack, Leon. See *Wriston, Lack, and Shemin,* 603
Lambert, G. Frederick. See *Rose, Coon, Lockhart, and Lambert,* 101
Lardy, Henry A. See *Maley and Lardy,* 377
Larner, Joseph, and **McNickle, C. M.** Gastrointestinal digestion of starch. I. The action of oligo-1,6-glucosidase on branched saccharides, 723
Laskowski, M. See *Privat de Garilhe and Laskowski,* 269
Lehninger, Albert L. See *Borgström, Sudduth, and Lehninger,* 571
—. See *Nielsen and Lehninger,* 555
Lentz, Kenneth, and **Wood, Harland G.** Synthesis of acetate from formate and carbon dioxide by *Clostridium thermoaceticum,* 645
—. See *Bernstein, Lentz, Malm, Schambye, and Wood,* 137
Leon, Arthur S. See *Deiss and Leon,* 685
Levintow, Leon. See *Meister, Levintow, Greenfield, and Abendschein,* 441
Li, Choh Hao. See *Raacke and Li,* 277
Lieberman, Irving, Kornberg, Arthur, and **Simms, Ernest S.** Enzymatic synthesis of nucleoside diphosphates and triphosphates, 429
—, —, and —. Enzymatic synthesis of pyrimidine nucleotides. Orotidine-5'-phosphate and uridine-5'-phosphate, 403
—. See *Kornberg, Lieberman, and Simms,* 389, 417
Liener, Irvin E. See *Viswanatha and Liener,* 777
Lindsley, John. See *Troll and Lindsley,* 655
Lipshitz, Rakoma. See *Spitnik, Lipshitz, and Chargaff,* 765
Lockhart, Haines B. See *Rose, Coon, Lockhart, and Lambert,* 101
Lorber, Victor, and **Cook, Margaret.** The metabolism of *n*-butyrate-C^{14} by mammalian heart muscle, 823

XUM

Lott, Mary Harriet. See *Huffman and Lott*, 627, 633

Lowy, Peter H. See *Borsook, Abrams, and Lowy*, 111

M

MacKinnon, Joan A. See *Bueding and MacKinnon*, 495, 507

Maley, Gladys Feldott, and **Lardy, Henry A.** Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rat livers, 377

Malm, Mignon. See *Bernstein, Lentz, Malm, Schambye, and Wood*, 137

Matsuo, Yoshihiko, and **Greenberg, David M.** Metabolic formation of homoserine and α-aminobutyric acid from methionine, 547

McNickle, C. M. See *Larner and McNickle*, 723

Meister, Alton, Levintow, Leon, Greenfield, Robert E., and **Abendschein, Patricia A.** Hydrolysis and transfer reactions catalyzed by ω-amidase preparations, 441

Moore, Stanford. See *Crampton, Moore, and Stein*, 787

Morgan, Joseph F., and **Morton, Helen J.** Studies on the sulfur metabolism of tissues cultivated *in vitro*. I. A critical requirement of L-cystine, 539

Morita, Toshiko N. See *Nakada, Morita, and Wick*, 803

Morton, Helen J. See *Morgan and Morton*, 539

Mounter, L. A. The complex nature of dialkylfluorophosphatases of hog and rat liver and kidney, 705

—, **Baxter, Robert F.,** and **Chanutin, Alfred.** Dialkylfluorophosphatases of microorganisms, 699

—, **Dien, Lien Tien H.,** and **Chanutin, Alfred.** The distribution of dialkylfluorophosphatases in the tissues of various species, 691

N

Nakada, Henry I., Morita, Toshiko N., and **Wick, Arne N.** Studies on the relationships between insulin, glucosamine, and glucose in rat diaphragms, 803

Nakayama, Teishi. See *Okuhara and Nakayama*, 295

Nesbett, Frances B. See *Ashmore, Renold, Nesbett, and Hastings*, 153

Nielsen, Sigurd Olaf, and **Lehninger, Albert L.** Phosphorylation coupled to the oxidation of ferrocytochrome *c*, 555

Nyc, Joseph F., and **Zabin, Irving.** The formation of serine from pyruvate, 35

O

O'Kane, D. J. See *Wolfe and O'Kane*, 637

Okuhara, Eiji, and **Nakayama, Teishi.** Studies on the conjugated lipides. VI. On the structure of inositol phospholipide, 295

P

Perlman, D. Spectrophotometric method for the determination of 5-keto-D-gluconic acid, 353

Pihl, Alexander, and **Fritzson, Per.** The catabolism of C^{14}-labeled β-alanine in the intact rat, 345

Potter, Van R. See *Aisenberg and Potter*, 737

—. See *Siekevitz and Potter*, 221, 237

Privat de Garilhe, M., and **Laskowski, M.** Study of the enzymatic degradation of deoxyribonucleic acid by two different deoxyribonucleodepolymerases, 269

Prusoff, William H. Studies on the mechanism of action of 6-azathymine. I. Biosynthesis of the deoxyriboside, 809

R

Raacke, I. D., and **Li, Choh Hao.** Corticotropins (ACTH). VI. Isoelectric point of α-corticotropin as determined by zone electrophoresis on starch, 277

Rachele, Julian R., Kuchinskas, Edward J., Kratzer, F. Howard, and **du**

XUM

Vigneaud, Vincent. Hydrogen isotope effect in the oxidation *in vivo* of methionine labeled in the methyl group, 593

Renold, Albert E. See *Ashmore, Renold, Nesbett, and Hastings*, 153

Rice, Louise. See *Touster, Hutcheson, and Rice*, 677

Roe, Joseph H. See *Futterman and Roe*, 257

Rose, William C., Coon, Minor J., Lockhart, Haines B., and **Lambert, G. Frederick.** The amino acid requirements of man. XI. The threonine and methionine requirements, 101

Rosenkrantz, Harris. See *Forchielli, Rosenkrantz, and Dorfman*, 713

Russell, Charlotte S. See *Shemin, Russell, and Abramsky*, 613

S

Sable, Henry Z. See *Guarino and Sable*, 515

Schaffner, G. See *Woolley, Schaffner, and Braun*, 485

Schambye, Per. See *Bernstein, Lentz, Malm, Schambye, and Wood*, 137

Schmidt, Gerhard. See *Thannhauser, Fellig, and Schmidt*, 211

Serlin, Irving, and **Cotzias, George C.** Microdiffusion of acetic acid as an assay for acetylcholinesterase, 263

Shemin, David, Russell, Charlotte S., and **Abramsky, Tessa.** The succinate-glycine cycle. I. The mechanism of pyrrole synthesis, 613

—. See *Wriston, Lack, and Shemin*, 603

Shuster, Louis, and **Kaplan, Nathan O.** The preparation and properties of 3′-triphosphopyridine nucleotide, 183

—, —, and **Stolzenbach, Francis E.** The reaction of pyridine nucleotides with trifluoroacetic acid anhydride, 195

Siekevitz, Philip, and **Potter, Van R.** Biochemical structure of mitochondria. I. Intramitochondrial components and oxidative phosphorylation, 221

Siekevitz, Phillip, and **Potter, Van R.** II. Radioactive labeling of intramitochondrial nucleotides during oxidative phosphorylation, 237

Sime, John T., and **Johnson, B. Connor.** Metabolism of methionine-$C^{14}H_3$ in the chick. II. Distribution of C^{14} in the urinary uric acid molecule, 41

Simms, Ernest S. See *Kornberg, Lieberman, and Simms*, 389, 417

—. See *Lieberman, Kornberg, and Simms*, 403, 429

Sinsheimer, Robert L. The action of pancreatic deoxyribonuclease. II. Isomeric dinucleotides, 579

Smith, Emil L., Kimmel, J. R., Brown, Douglas M., and **Thompson, E. O. P.** Isolation and properties of a crystalline mercury derivative of a lysozyme from papaya latex, 67

—. See *Dannenberg and Smith*, 45, 55

Smith, Lucile. Reactions of cytochromes *a* and a_3. I. Studies of oxidation and reduction of the pigments in a purified preparation, 833

—. II. Studies with *Micrococcus pyogenes* var. *albus* and *Bacillus subtilis*, 847

Spitnik, Pnina, Lipshitz, Rakoma, and **Chargaff, Erwin.** Studies on nucleoproteins. III. Deoxyribonucleic acid complexes with basic polyelectrolytes and their fractional extraction, 765

Stein, William H. See *Crampton, Moore, and Stein*, 787

Stetten, DeWitt, Jr. See *Bloom, Eisenberg, and Stetten*, 461

Stewart, Charles J., Cheldelin, Vernon H., and **King, Tsoo E.** Synthesis and biological activities of pantetheine analogues. XVI. Pantothenic acid studies, 319

Stolzenbach, Francis E. See *Shuster, Kaplan, and Stolzenbach*, 195

Sudduth, Herschel C. See *Borgström, Sudduth, and Lehninger*, 571

T

Thannhauser, S. J., Fellig, Josef, and **Schmidt, Gerhard.** The structure of

cerebroside sulfuric ester of beef brain, 211

Thomasson, Joan. See *Gabrio, Hennessey, Thomasson, and Finch*, 357

Thompson, E. O. P. See *Smith, Kimmel, Brown, and Thompson*, 67

Tolbert, N. E. Formic acid metabolism in barley leaves, 27

Tong, W., and **Chaikoff, I. L.** Metabolism of I^{131} by the marine alga, *Nereocystis luetkeana*, 473

Touster, Oscar, Hutcheson, Ruth M., and **Rice, Louise.** The influence of D-glucuronolactone on the excretion of L-xylulose by humans and guinea pigs, 677

Troll, Walter, and **Lindsley, John.** A photometric method for the determination of proline, 655

U

Udenfriend, Sidney, Weissbach, Herbert, and **Clark, Carroll T.** The estimation of 5-hydroxytryptamine (serotonin) in biological tissues, 337

V

Varner, J. E. See *Webster and Varner*, 91

du Vigneaud, Vincent. See *Rachele, Kuchinskas, Kratzer, and du Vigneaud*, 593

Villee, Claude A. An estradiol-induced stimulation of citrate utilization by placenta, 171

Viswanatha, T., and **Liener, Irvin E.** The inhibition of trypsin. III. Influence of urea, 777

W

Warner, Robert C. See *Fresco and Warner*, 751

Webster, George C., and **Varner, J. E.** Aspartate metabolism and asparagine synthesis in plant systems, 91

Weissbach, Herbert. See *Udenfriend, Weissbach, and Clark*, 337

West, Edward S. See *Beatty and West*, 661

Wick, Arne N. See *Nakada, Morita, and Wick*, 803

Wolfe, R. S., and **O'Kane, D. J.** Cofactors of the carbon dioxide exchange reaction of *Clostridium butyricum*, 637

Wood, Harland G. See *Bernstein, Lentz, Malm, Schambye, and Wood*, 137

—. See *Lentz and Wood*, 645

Woolley, D. W., Schaffner, G., and **Braun, Armin C.** Studies on the structure of the phytopathogenic toxin of *Pseudomonas tabaci*, 485

Wriston, John C., Jr., Lack, Leon, and **Shemin, David.** The mechanism of porphyrin formation. Further evidence on the relationship of the citric acid cycle and porphyrin formation, 603

Z

Zabin, Irving. See *Nyc and Zabin*, 35

INDEX TO SUBJECTS

A

Acetate: Activation, kidney and liver, fluoride and dinitrophenol effect, *Aisenberg and Potter*, 737
Synthesis, carbon dioxide relation, *Clostridium thermoaceticum, Lentz and Wood*, 645
—, formate relation, *Clostridium thermoaceticum, Lentz and Wood*, 645
Acetic acid: Acetylcholinesterase determination, relation, *Serlin and Cotzias*, 263
Trifluoro-. *See* Trifluoroacetic acid
Acetylcholinesterase: Determination, acetic acid relation, *Serlin and Cotzias*, 263
Alanine: β-, carbon 14-labeled, catabolism, *Pihl and Fritzson*, 345
Alga: Marine. *See also Nereocystis luetkeana*
Alloxan: Diabetes, glucose catabolism, Embden-Meyerhof pathway, *Bloom*, 467
—, ketosis, fructose effect, *Beatty and West*, 661
—, —, malic acid effect, *Beatty and West*, 661
—, —, succinic acid effect, *Beatty and West*, 661
Amidase: ω-, reactions, *Meister, Levintow, Greenfield, and Abendschein*, 441
Amino acid(s): Esters, polymers from, lung proteinase I, relation, *Dannenberg and Smith*, 55
Fructose-, liver, amino acid effect, *Borsook, Abrams, and Lowy*, 111
Requirements, *Rose, Coon, Lockhart, and Lambert*, 101
Aminobutyric acid: α-, formation *in vivo*, methionine relation, *Matsuo and Greenberg*, 547
Amino-β-hydroxycaproic acid(s): α-, synthetic, *Buston and Bishop*, 217
Androstan-3β,16α-diol: *Huffman and Lott*, 633
Androstan-3β,16β-diol: *Huffman and Lott*, 633
Asparagine: Synthesis, plant systems, *Webster and Varner*, 91
Aspartate: Metabolism, plant systems, *Webster and Varner*, 91
Azathymine: 6-, action, mechanism, *Prusoff*, 809
Azathymine deoxyriboside: 6-, biosynthesis, *Prusoff*, 809

B

Bacillus subtilis: Cytochromes *a* and a_3, *Smith*, 847
Bacteria: *See also Clostridium, Leuconostoc, Micrococcus, Pseudomonas*
Barley: Leaves, formic acid metabolism, *Tolbert*, 27
Blood cell: Red, preservation, *Gabrio, Hennessey, Thomasson, and Finch*, 357
Brain: Cerebroside sulfuric ester, chemical constitution, *Thannhauser, Fellig, and Schmidt*, 211
Butyrate: *n*-, carbon 14-labeled, metabolism, heart, *Lorber and Cook*, 823
Butyric acid: α-Amino-. *See* Aminobutyric acid

C

Caproic acid(s): α-Amino-β-hydroxy-. *See* Amino-β-hydroxycaproic acid
Carbohydrate: Metabolism, liver, *Ashmore, Renold, Nesbett, and Hastings*, 153
Carbon dioxide: Acetate synthesis from, *Clostridium thermoaceticum, Lentz and Wood*, 645
Exchange reaction, *Clostridium butyricum, Wolfe and O'Kane*, 637
Cerebroside sulfuric ester: Brain, chemical constitution, *Thannhauser, Fellig, and Schmidt*, 211
Citrate: Utilization, placenta, estradiol effect, *Villee*, 171

XUM

Citric acid: Cycle, porphyrin formation, relation, *Wriston, Lack, and Shemin*, 603
Clearing factor: *Korn*, 1, 15
Clostridium butyricum: Carbon dioxide exchange reaction, *Wolfe and O'Kane*, 637
Clostridium thermoaceticum: Acetate synthesis from formate and carbon dioxide, *Lentz and Wood*, 645
Coconut oil: Lipoprotein lipase, heparin-activated, *Korn*, 15
Corticotropin(s): *Raacke and Li*, 277
α-, isoelectric point, *Raacke and Li*, 277
Cortisol: Deoxy-. *See* Deoxycortisol
Cortisone: Metabolism, liver, *Eisenstein*, 585
Cystine: L-, requirement, tissue cultures, *Morgan and Morton*, 539
Cytochrome(s): *a*, *Bacillus subtilis*, *Smith*, 847
—, *Micrococcus pyogenes*, *Smith*, 847
—, reactions, *Smith*, 833, 847
a_3, *Bacillus subtilis*, *Smith*, 847
—, *Micrococcus pyogenes*, *Smith*, 847
—, reactions, *Smith*, 833, 847
c, reduction, phosphorylation and β-hydroxybutyrate relation, *Borgström, Sudduth, and Lehninger*, 571
Ferro-. *See* Ferrocytochrome

D

Deoxycortisol: 11-, metabolism, *in vitro*, *Forchielli, Rosenkrantz, and Dorfman*, 713
Deoxyribonuclease: Pancreas, *Sinsheimer*, 579
Deoxyribonucleic acid: Complexes, *Spitnik, Lipshitz, and Chargaff*, 765
Degradation, enzymatic, deoxyribonucleodepolymerase relation, *Privat de Garilhe and Laskowski*, 269
Deoxyribonucleodepolymerase(s): Deoxyribonucleic acid degradation, enzymatic, relation, *Privat de Garilhe and Laskowski*, 269
Diabetes: Alloxan, glucose catabolism, Embden-Meyerhof pathway, *Bloom*, 467
—, ketosis, fructose effect, *Beatty and West*, 661
—, —, malic acid effect, *Beatty and West*, 661
—, —, succinic acid effect, *Beatty and West*, 661
Glycerol metabolism, liver, *Ashmore, Renold, Nesbett, and Hastings*, 153
Dialkylfluorophosphatase(s): Kidney, *Mounter*, 705
Liver, *Mounter*, 705
Microorganisms, *Mounter, Baxter, and Chanutin*, 699
Tissues, *Mounter, Dien, and Chanutin*, 691
Diaphragm: Insulin, glucosamine, and glucose relationship, *Nakada, Morita, and Wick*, 803
Dinitrophenol: Acetate activation, kidney and liver, effect, *Aisenberg and Potter*, 737
Dinucleotide(s): Isomeric, pancreatic deoxyribonuclease action, *Sinsheimer*, 579

E

Embden-Meyerhof: Pathway, glucose catabolism, alloxan diabetes and fasting, *Bloom*, 467
Enzyme(s): Deoxyribonucleic acid degradation, deoxyribonucleodepolymerase relation, *Privat de Garilhe and Laskowski*, 269
Malonic acid decarboxylation, *Hayaishi*, 125
Nucleoside diphosphate synthesis, *Lieberman, Kornberg, and Simms*, 429
— triphosphate synthesis, *Lieberman, Kornberg, and Simms*, 429
Orotidine-5′-phosphate synthesis, *Lieberman, Kornberg, and Simms*, 403
Phosphate transfer, mechanism, *Harrison, Boyer, and Falcone*, 303
5-Phosphoribosylpyrophosphate synthesis, *Kornberg, Lieberman, and Simms*, 389

Enzyme(s)—*continued:*
Proteolysis, lung, *Dannenberg and Smith*, 45
Purine nucleotide synthesis, *Kornberg, Lieberman, and Simms*, 417
Pyrimidine nucleotide synthesis, *Lieberman, Kornberg, and Simms*, 403
Uridine-5′-phosphate synthesis, *Lieberman, Kornberg, and Simms*, 403
See also Acetylcholinesterase, Amidase, etc.
Erythrocyte: *See* Blood cell, red
Esterase: Acetylcholin-. *See* Acetylcholinesterase
Estradiol: -3,16α. *Huffman and Lott*, 627
Placenta citrate utilization, effect, *Villee*, 171

F

Fasting: Glucose catabolism, Embden-Meyerhof pathway, *Bloom*, 467
Ferrocytochrome: *c*, oxidation, phosphorylation relation, *Nielsen and Lehninger*, 555
Fluoride: Acetate activation, kidney and liver, effect, *Aisenberg and Potter*, 737
Formate: Acetate synthesis from, *Clostridium thermoaceticum, Lentz and Wood*, 645
Formic acid: Metabolism, leaves, barley, *Tolbert*, 27
Fructose: -Amino acids, liver, amino acid effect, *Borsook, Abrams, and Lowy*, 111
Ketosis, alloxan diabetes, effect, *Beatty and West*, 661

G

Gastrointestinal tract: Digestion, saccharides, branched, oligo-1,6-glucosidase effect, *Larner and McNickle*, 723
—, starch, *Larner and McNickle*, 723
Glomerulus: Urine, potassium, *Necturus, Bott*, 287
Gluconic acid: 5-Keto-D-. *See* Keto-D-gluconic acid
Glucosamine: Diaphragm, effect, *Nakada, Morita, and Wick*, 803
Glucose: Carbon 14-labeled, degradation, *Leuconostoc mesenteroides, Bernstein, Lentz, Malm, Schambye, and Wood*, 137
Catabolism, Embden-Meyerhof pathway, alloxan diabetes and fasting, *Bloom*, 467
—, liver, phosphogluconate oxidation pathway relation, *Bloom, Eisenberg, and Stetten*, 461
Diaphragm, effect, *Nakada, Morita, and Wick*, 803
Glucosidase: Oligo-1,6-. *See* Oligo-1,6-glucosidase
Glucuronic acid: Glucuronide. *See* Glucuronide glucuronic acid
Metabolism, glucuronide glucuronic acid determination, relation, *Fishman and Green*, 527
Glucuronidase: β-, action, glucuronide glucuronic acid determination, relation, *Fishman and Green*, 527
Glucuronide glucuronic acid: Determination, β-glucuronidase action, relation, *Fishman and Green*, 527
—, glucuronic acid studies, relation, *Fishman and Green*, 527
Glucuronolactone: D-, L-xylulose excretion, effect, *Touster, Hutcheson, and Rice*, 677
Glycerol: Metabolism, liver, diabetes, *Ashmore, Renold, Nesbett, and Hastings*, 153
Glycine: -Succinate cycle, pyrrole synthesis, relation, *Shemin, Russell, and Abramsky*, 613

H

Heart: *n*-Butyrate-C^{14} metabolism, *Lorber and Cook*, 823
Lipoprotein lipase, heparin-activated, *Korn*, 1
Valve mucoprotein mucopolysaccharides, *Deiss and Leon*, 685
Heparin: -Activated lipoprotein lipase, coconut oil, *Korn*, 15
— — —, heart, *Korn*, 1
Hexokinase(s): *Schistosoma mansoni, Bueding and MacKinnon*, 495

Histone: Thymus, fractionation, chromatographic, *Crampton, Moore, and Stein,* 787
Homoserine: Formation *in vivo,* methionine relation, *Matsuo and Greenberg,* 547
Hydrogen: Isotope, methionine, methyl group-labeled, oxidation, *in vivo,* effect, *Rachele, Kuchinskas, Kratzer, and du Vigneaud,* 593
Hydroxybutyrate: β-, cytochrome *c* reduction, relation, *Borgström, Sudduth, and Lehninger,* 571
Hydroxytryptamine: 5-, determination, *Udenfriend, Weissbach, and Clark,* 337

I

Inositol phospholipide: Chemical constitution, *Okuhara and Nakayama,* 295
Insulin: Diaphragm, effect, *Nakada, Morita, and Wick,* 803
Hydrolysis, lung proteinase I, relation, *Dannenberg and Smith,* 55
Iodine: Isotopic, metabolism, *Nereocystis luetkeana, Tong and Chaikoff,* 473
Isomerase: Phosphoglucose. *See* Phosphoglucose isomerase

K

Keto-D-gluconic acid: 5-, determination, spectrophotometric, *Perlman,* 353
Ketosis: Diabetes, alloxan, fructose effect, *Beatty and West,* 661
—, —, malic acid effect, *Beatty and West,* 661
—, —, succinic acid effect, *Beatty and West,* 661
Kidney: Acetate activation, fluoride and dinitrophenol effect, *Aisenberg and Potter,* 737
Dialkylfluorophosphatase, *Mounter,* 705

L

Latex: Papaya, lysozyme, crystalline mercury derivative, isolation and properties, *Smith, Kimmel, Brown, and Thompson,* 67
Leuconostoc mesenteroides: Glucose-C^{14} degradation, *Bernstein, Lentz, Malm, Schambye, and Wood,* 137
Lipase: Lipoprotein, heparin-activated, coconut oil, *Korn,* 15
—, —, heart, *Korn,* 1
Lipide(s): Conjugated, *Okuhara and Nakayama,* 295
Lipoprotein: Lipase, heparin-activated, coconut oil, *Korn,* 15
—, —, heart, *Korn,* 1
Liver: Acetate activation, fluoride and dinitrophenol effect, *Aisenberg and Potter,* 737
Carbohydrate metabolism, *Ashmore, Renold, Nesbett, and Hastings,* 153
Cortisone metabolism, *Eisenstein,* 585
Dialkylfluorophosphatase, *Mounter,* 705
Fructose-amino acids, amino acid effect, *Borsook, Abrams, and Lowy,* 111
Glucose catabolism, phosphogluconate oxidation pathway relation, *Bloom, Eisenberg, and Stetten,* 461
Glycerol metabolism, diabetes, *Ashmore, Renold, Nesbett, and Hastings,* 153
Mitochondria, oxidation, thyrotoxicosis, phosphorylation relation, *Maley and Lardy,* 377
Lung: Enzymes, proteolytic, *Dannenberg and Smith,* 45
Proteinase I, insulin hydrolysis, relation, *Dannenberg and Smith,* 55
— —, polymers from amino acid esters, relation, *Dannenberg and Smith,* 55
Lymph: Organ, protease, *Hess, Campbell, and Herranen,* 163
Lysozyme: Crystalline mercury derivative, isolation and properties, papaya latex, *Smith, Kimmel, Brown, and Thompson,* 67

M

Malic acid: Ketosis, alloxan diabetes, effect, *Beatty and West,* 661

Malonic acid: Decarboxylation, enzymatic, *Hayaishi*, 125
Methionine: α-Aminobutyric acid formation from, *in vivo*, *Matsuo and Greenberg*, 547
Carbon 14-labeled, metabolism, urine uric acid, relation, *Sime and Johnson*, 41
Homoserine formation from, *in vivo*, *Matsuo and Greenberg*, 547
Methyl group-labeled, oxidation, *in vivo*, hydrogen isotope effect, *Rachele, Kuchinskas, Kratzer, and du Vigneaud*, 593
Requirement, *Rose, Coon, Lockhart, and Lambert*, 101
Micrococcus pyogenes: Cytochromes *a* and a_3, *Smith*, 847
Microorganism(s): Dialkylfluorophosphatase, *Mounter, Baxter, and Chanutin*, 699
See also Bacillus, Bacteria
Mitochondrion: Liver, oxidation, thyrotoxicosis, phosphorylation relation, *Maley and Lardy*, 377
Nucleotides, radioactive labeling, phosphorylation, oxidative, relation, *Siekevitz and Potter*, 237
Phosphorylation, oxidative, relation, *Siekevitz and Potter*, 221
Structure, biochemical, *Siekevitz and Potter*, 221, 237
Mucopolysaccharide(s): Heart valve, mucoprotein, *Deiss and Leon*, 685
Mucoprotein: Heart valve, mucopolysaccharides, *Deiss and Leon*, 685
Muscle: *See also* Diaphragm, Heart
Mutase: Phospho-. *See* Phosphomutase
Phosphogluco-. *See* Phosphoglucomutase
Phosphoribo-. *See* Phosphoribomutase

N

Necturus: Urine, glomerulus, potassium, *Bott*, 287
Nereocystis luetkeana: Iodine, isotopic, metabolism, *Tong and Chaikoff*, 473
Nuclease: Deoxyribo-. *See* Deoxyribonuclease
Nucleic acid: Deoxyribo-. *See* Deoxyribonucleic acid
Nucleoprotein(s): *Spitnik, Lipshitz, and Chargaff*, 765
Nucleoside diphosphate(s): Synthesis, enzymatic, *Lieberman, Kornberg, and Simms*, 429
Nucleoside triphosphate(s): Synthesis, enzymatic, *Lieberman, Kornberg, and Simms*, 429
Nucleotide(s): Mitochondria, radioactive labeling, phosphorylation, oxidative, relation, *Siekevitz and Potter*, 237
Purine. *See* Purine nucleotide
Pyridine. *See* Pyridine nucleotide
Pyrimidine. *See* Pyrimidine nucleotide
Shock, tourniquet, *Jordan and Gray*, 669
3′-Triphosphopyridine. *See* Triphosphopyridine nucleotide

O

Oil: *See also* Coconut oil
Oligo-1,6-glucosidase: Saccharides, branched, digestion, effect, *Larner and McNickle*, 723
Orotidine-5′-phosphate: Synthesis, enzymatic, *Lieberman, Kornberg, and Simms*, 403

P

Pancreas: Deoxyribonuclease, dinucleotides, isomeric, action, *Sinsheimer*, 579
Pantetheine: Analogues, synthesis and metabolism, *Stewart, Cheldelin, and King*, 319
Pantothenic acid: *Stewart, Cheldelin, and King*, 319
Papaya: Latex, lysozyme crystalline mercury derivative, isolation and properties, *Smith, Kimmel, Brown, and Thompson*, 67
Phenol: Dinitro-. *See* Dinitrophenol
Phosphatase(s): Dialkylfluoro-. *See* Dialkylfluorophosphatase
Phosphate: Transfer, enzymatic, mechanism, *Harrison, Boyer, and Falcone*, 303

Phosphoglucomutase: *Guarino and Sable*, 515
Phosphogluconate: Oxidation pathway, liver glucose catabolism, relation, *Bloom, Eisenberg, and Stetten*, 461
Phosphoglucose isomerase: *Schistosoma mansoni, Bueding and MacKinnon*, 507
Phospholipide: Inositol. *See* Inositol phospholipide
Phosphomutase(s): *Guarino and Sable*, 515
Phosphoribomutase: *Guarino and Sable*, 515
Phosphoribosylpyrophosphate: 5-, synthesis, enzymatic, and properties, *Kornberg, Lieberman, and Simms*, 389
Phosphorylation: Cytochrome *c* reduction, relation, *Borgström, Sudduth, and Lehninger*, 571
Ferrocytochrome *c* oxidation, relation, *Nielsen and Lehninger*, 555
Liver mitochondria, oxidation, thyrotoxicosis, *Maley and Lardy*, 377
Oxidative, mitochondria, relation, *Siekevitz and Potter*, 221
—, mitochondrial nucleotides, radioactive labeling, relation, *Siekevitz and Potter*, 237
Placenta: Citrate utilization, estradiol effect, *Villee*, 171
Plant: Systems, asparagine synthesis, *Webster and Varner*, 91
—, aspartate metabolism, *Webster and Varner*, 91
Polymerase(s): Deoxyribonucleode-. *See* Deoxyribonucleodepolymerase
Polysaccharide(s): Muco-. *See* Mucopolysaccharide
Porphyrin: Formation, citric acid cycle relation, *Wriston, Lack, and Shemin*, 603
Potassium: Urine, glomerulus, *Necturus, Bott*, 287
Proline: Determination, photometric, *Troll and Lindsley*, 655
Protease: Lymph organ, *Hess, Campbell, and Herranen*, 163
Protein(s): Lipo-. *See* Lipoprotein
Muco-. *See* Mucoprotein
Nucleo-. *See* Nucleoprotein
Proteinase: I, lung, insulin hydrolysis, relation, *Dannenberg and Smith*, 55
—, —, polymers from amino acid esters, relation, *Dannenberg and Smith*, 55
Proteolysis: Enzymes, lung, *Dannenberg and Smith*, 45
Pseudomonas tabaci: Toxin, phytopathogenic, chemical constitution, *Woolley, Schaffner, and Braun*, 485
Purine nucleotide(s): Synthesis, enzymatic, *Kornberg, Lieberman, and Simms*, 417
Pyridine nucleotide(s): Trifluoroacetic acid anhydride and, reaction, *Shuster, Kaplan, and Stolzenbach*, 195
Pyrimidine(s): Determination, isotope derivative method, *Fresco and Warner*, 751
Pyrimidine nucleotide(s): Synthesis, enzymatic, *Lieberman, Kornberg, and Simms*, 403
Pyrrole: Synthesis, succinate-glycine cycle, relation, *Shemin, Russell, and Abramsky*, 613
Pyruvate: Serine formation from, *Nyc and Zabin*, 35

R

Riboflavin: Tryptophan metabolism, relation, *Henderson, Koski, and D'Angeli*, 369
Ribonucleic acid: Deoxy-. *See* Deoxyribonucleic acid
Riboside: 6-Azathymine deoxy-. *See* Azathymine deoxyriboside
Ribulose: Urine, identification, *Futterman and Roe*, 257

S

Saccharide(s): Branched, digestion, oligo-1,6-glucosidase effect, *Larner and McNickle*, 723

Schistosoma mansoni: Hexokinase, *Bueding and MacKinnon*, 495
Phosphoglucose isomerase, *Bueding and MacKinnon*, 507
Serine: Formation, pyruvate relation, *Nyc and Zabin*, 35
Serotonin: *See also* Hydroxytryptamine
Shock: Tourniquet, nucleotides, *Jordan and Gray*, 669
Starch: Digestion, gastrointestinal, *Larner and McNickle*, 723
Steroid(s): 16-Substituted, *Huffman and Lott*, 627, 633
Succinate: -Glycine cycle, pyrrole synthesis, relation, *Shemin, Russell, and Abramsky*, 613
Succinic acid: Ketosis, alloxan diabetes, effect, *Beatty and West*, 661
Sulfur: Metabolism, tissue cultures, *Morgan and Morton*, 539

T

Threonine: Requirement, *Rose, Coon, Lockhart, and Lambert*, 101
Thymine: 6-Aza-. *See* Azathymine
Thymus: Histone, fractionation, chromatographic, *Crampton, Moore, and Stein*, 787
Thyrotoxicosis: Liver mitochondria, oxidation, phosphorylation relation, *Maley and Lardy*, 377
Tissue culture: L-Cystine requirement, *Morgan and Morton*, 539
Sulfur metabolism, *Morgan and Morton*, 539
Toxin: Phytopathogenic, *Pseudomonas tabaci*, chemical constitution, *Woolley, Schaffner, and Braun*, 485
Trauma: *Jordan and Gray*, 669
Trifluoroacetic acid: Anhydride, pyridine nucleotide and, reaction, *Shuster, Kaplan, and Stolzenbach*, 195
Triphosphopyridine nucleotide: 3'-, preparation and properties, *Shuster and Kaplan*, 183
Trypsin: Inhibition, urea effect, *Viswanatha and Liener*, 777
Tryptamine: Hydroxy-. *See* Hydroxytryptamine
Tryptophan: Metabolism, riboflavin relation, *Henderson, Koski, and D'Angeli*, 369
—, vitamin B_6 relation, *Henderson, Koski, and D'Angeli*, 369

U

Urea: Trypsin inhibition, effect, *Viswanatha and Liener*, 777
Uric acid: Urine, methionine-$C^{14}H_3$ metabolism, relation, *Sime and Johnson*, 41
Uridine-5'-phosphate: Synthesis, enzymatic, *Lieberman, Kornberg, and Simms*, 403
Urine: Glomerulus, potassium, *Necturus, Bott*, 287
Ribulose, identification, *Futterman and Roe*, 257
Uric acid, methionine-$C^{14}H_3$ metabolism, relation, *Sime and Johnson*, 41
L-Xylulose, identification, *Futterman and Roe*, 257

V

Vitamin: B_2. *See also* Riboflavin
B_6, tryptophan metabolism, relation, *Henderson, Koski, and D'Angeli*, 369

X

Xylulose: L-, excretion, D-glucuronolactone effect, *Touster, Hutcheson, and Rice*, 677
—, urine, identification, *Futterman and Roe*, 257

XUM